



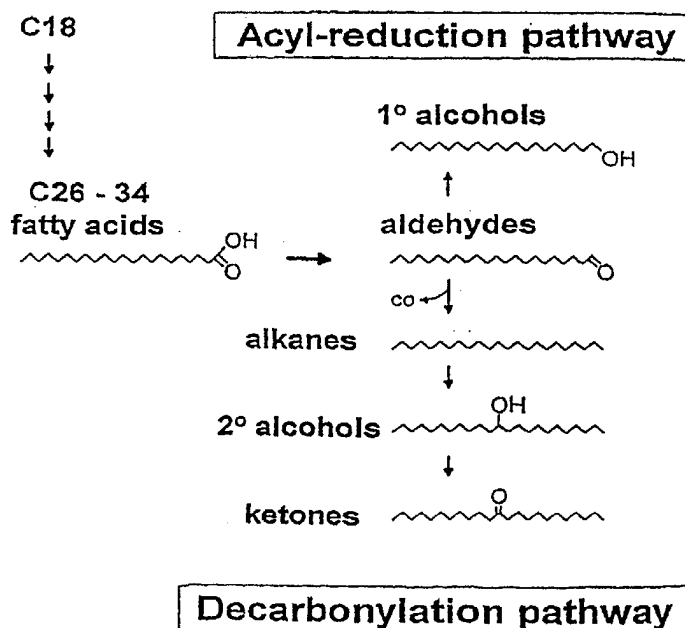
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/CA98/00343 <b>(22) International Filing Date:</b> 14 April 1998 (14.04.98)  <b>(30) Priority Data:</b> 60/043,831           14 April 1997 (14.04.97)       US 09/958,947       10 April 1998 (10.04.98)       US  <b>(71) Applicant:</b> THE UNIVERSITY OF BRITISH COLUMBIA [CA/CA]; I.R.C. Building, Room 331, 2194 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3 (CA).  <b>(72) Inventors:</b> KUNST, Ljerka; 2810 Newmarket Drive, North Vancouver, British Columbia V7R 2T4 (CA). MILLAR, Anthony, A.; 110-1235 Nelson Street, North Vancouver, British Columbia V6E 1J5 (CA).  <b>(74) Agent:</b> RAE, Patricia, A.; Sim & McBurney, 6th floor, 330 University Avenue, Toronto, Ontario M5G 1R7 (CA).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>

(54) Title: NUCLEIC ACIDS ENCODING A PLANT ENZYME INVOLVED IN VERY LONG CHAIN FATTY ACID SYNTHESIS

## (57) Abstract

Nucleic acid molecules encoding an enzyme involved in very long chain fatty acid (VLCFA) elongation in plants are disclosed. The invention includes a cDNA, genomic clone and encoded protein, as well as plants having modified VLCFA composition, such as modified epicuticular waxes, and methods of making such plants.

Arabidopsis Wax Biosynthesis

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## NUCLEIC ACIDS ENCODING A PLANT ENZYME INVOLVED IN VERY LONG CHAIN FATTY ACID SYNTHESIS

5

### Technical Field

This invention relates to DNA molecules cloned from plants and methods of using such DNA molecules to produce transgenic plants with altered fatty acid composition.

### Background

10       Epicuticular waxes form the outermost layer of the aerial portion of the plant and are thus the first line of interaction between the plant and its environment. The physical properties of this wax layer protect the plant from numerous environmental stresses. For example, the hydrophobic nature of wax prevents dehydration (nonstomatal water loss) and aids in shedding rainwater. The reflective nature of wax protects the plant against UV radiation (Reicosky and Hanover, 1978). Waxes are also known to  
15       protect against acid rain (Percy and Baker, 1990) and, because they are a good solvent for organic pollutants, they are able to impede the uptake of aqueous foliar sprays (Schreiber and Schonherr, 1992). Furthermore, surface waxes protect plants from bacterial and fungal (Jenks et al., 1994) pathogens and play a role in plant-insect interactions (Eigenbrode and Espelie, 1995). Recently it has been shown that some of the compounds found in epicuticular waxes are also present in the tryphine layer of pollen grains  
20       (Preuss et al., 1993). Without these compounds the tryphine layer erodes, resulting in pollen that is unable to function causing male sterility.

      Epicuticular waxes are composed of long chain, hydrophobic compounds all derived from saturated very long chain fatty acids (VLCFAs), that are synthesized within and then secreted from the epidermis. VLCFAs are defined as those fatty acids whose chain length is 20 or more carbons long.  
25       The lengths will vary from plant to plant, but typically, the wax VLCFAs are approximately 26-34 carbon long. These VLCFAs are synthesized by a microsomal fatty acid elongation (FAE) system by sequential additions of C2 moieties from malonyl-coenzyme A (CoA) to pre-existing fatty acids derived from the *de novo* fatty acid synthesis (FAS) pathway of the plastid. Analogous to *de novo* FAS it is thought that each cycle of FAE involves four enzymatic reactions; (1) condensation of malonyl-CoA with  
30       a long chain acyl-CoA, (2) reduction to  $\beta$ -hydroxyacyl-CoA, (3) dehydration to an enoyl-CoA and (4) reduction of the enoyl-CoA, resulting in the elongated acyl-CoA (Fehling and Mukherjee, 1991). Together these four activities are termed the elongase (von Wettstein-Knowles, 1982). VLCFAs in the epidermis are then converted to the other wax components through a number of pathways consisting of multienzyme complexes. For example VLCFAs are converted to aldehydes by fatty acyl-CoA reductase  
35       (Kolattukudy, 1971). These aldehydes can either be reduced by aldehyde reductase to produce primary alcohols (Kolattukudy, 1971), or decarbonylated by an aldehyde decarbonylase to produce odd chained alkanes (Cheesbrough and Kolattukudy, 1984). Alkanes can then undergo oxidation to form firstly

secondary alcohols and then ketones (for review see Post-Beittenmiller, 1996). Very little is known at the molecular level about the components that are involved in the biosynthesis of wax specific compounds and their secretion onto the plant surface. Genetic studies have shown that there are a large number of genes involved in these processes (for example, 22 loci have been reported in *Arabidopsis*, 84  
5 in barley). However only a few of these genes have been isolated so far and the biochemical role of their gene products remains unknown (Lemieux, 1996).

In addition to being made in the epidermal cells, VLCFAs also accumulate in the seed oil of some plant species. To date, developing seeds have been the primary focus of research into VLCFA biosynthesis. In seeds VLCFAs are incorporated into triacylglycerols (TAGs), as in the *Brassicaceae*, or  
10 into wax esters, as in Jojoba. The seed VLCFAs include the agronomically important erucic acid (C22:1), with oils containing this fatty acid used in the manufacture of lubricants, nylon, cosmetics, pharmaceuticals and plasticisers (Battey et al., 1989); Johnston and Fritz, 1989). Conversely, VLCFAs have detrimental nutritional effects and are therefore undesirable in edible oils. This has led to the breeding of Canola rapeseed varieties that are almost devoid of VLCFAs (Stefansson et al., 1961).

15 The seeds of *Arabidopsis* contain approximately 28% [w/wt of total fatty acids (FA)] of VLCFAs, eicosenoic acid (20:1) being the predominant VLCFA (21% of wt/wt of total FA). To identify the gene products that are involved in the synthesis of seed VLCFAs and establish the VLCFA biosynthetic pathway, several groups performed mutational analysis and screened for seed that had reduced VLCFA content. Each group independently identified the *FATTY ACID ELONGATION1* gene  
20 (*FAE1*; James and Dooner, 1990; Kunst et al., 1992; Lemieux et al., 1990). A mutation at this locus resulted in reduced VLCFA levels (<1% wt/wt of total FA) in the seed. Several other mutations that were non-allelic to *FAE1* were also isolated. However, these mutations had a less pronounced effect in that VLCFAs still constituted 6.7% (wt/wt of total FA) of the seed fatty acid (Katavic et al., 1995; Kunst et al., 1992). Thus, despite the fact that four enzymatic activities are required for each elongation  
25 step, the *FAE1* gene was the only one found by mutant analysis that resulted in almost complete loss of VLCFA synthesis in the seed.

The *Arabidopsis FAE1* gene was subsequently cloned (James et al., 1995; WO 96/13582), and showed homology to three condensing enzymes: chalcone synthase, stilbene synthase and  $\beta$ -ketoacyl-  
[acyl carrier protein] synthase III (17 amino acids were identical to a 50 amino acid region of a  
30 consensus sequence for condensing enzymes). Based on this homology it was proposed that *FAE1* encodes a  $\beta$ -ketoacyl-coenzyme A synthase (KCS), the condensing enzyme which catalyzes the first reaction of the microsomal fatty acid elongation system (James et al., 1995). As determined by Northern analysis, the *FAE1* gene is expressed in seeds of *Arabidopsis*, but is absent from leaves (James et al., 1995). This result is consistent with the fact that the *fae1* mutation affects only the fatty acid  
35 composition of the developing seed, having no pleiotropic effects on fatty acid composition of the vegetative, or floral parts of the plant. Thus, *FAE1* is regarded as a seed-specific condensing enzyme.

Recently a cDNA from Jojoba seeds involved in the syntheses of VLCFAs has been isolated (Lassner et al., 1996; WO 95/15387). The protein encoded by this cDNA showed high homology to

FAE1 (52% amino acid identity), and biochemical analysis demonstrated that it has a KCS activity. Using Jojoba KCS cDNA, Lassner et al. (1996) were able to complement the mutation in a Canola variety of *Brassica napus*, restoring a low erucic acid rapeseed line to a line that contained higher levels of VLCFAs. This suggests that in Canola, the mutation is in the structural gene encoding KCS, or a  
 5 gene affecting KCS activity. Thus, both in *Arabidopsis* and *Brassica napus*, the mutations that result in the abolition of VLCFA synthesis seem to affect the condensing enzyme.

If four enzyme activities are necessary for an elongation step, and *FAE1* and Jojoba-KCS only encode the KCS activity, one might expect to find other complementation groups that result in very low levels of VLCFAs synthesis. Because these complementation groups were not found in mutation  
 10 screenings, Millar and Kunst (1997) have hypothesized that these three activities are not seed specific, but ubiquitously present throughout the plant and shared with other FAE systems involved in VLCFA formation including wax biosynthesis. To test this *FAE1* was ecotopically expressed in yeast and in tissues of *Arabidopsis* and tobacco, where significant quantities of VLCFAs are not found. Expression of *FAE1* alone in these cells resulted in the biosynthesis and accumulation of VLCFAs. This  
 15 demonstrated that the condensing enzyme is the pivotal control point of the elongase, controlling not only the amounts of VLCFAs produced, but also their chain lengths. In contrast, it appears that the other three enzyme activities of the elongase are found ubiquitously throughout the plant, are not rate limiting and play no role in the control of VLCFA synthesis. The ability of yeast containing *FAE1* to synthesize VLCFAs suggests that the expression, and the acyl chain length specificity of the condensing enzyme,  
 20 along with the apparent broad specificities of the other three FAE activities, may be universal eukaryotic mechanism for regulating the amounts and acyl chain length of VLCFAs synthesized in any given cell (Millar and Kunst, 1997).

Thus, considering the central role of the condensing enzyme for VLCFA synthesis, the isolation of genes encoding condensing enzymes involved in the production of wax specific VLCFAs would  
 25 facilitate the modification of wax composition through genetic engineering. Furthermore, since the majority of wax components are derived from VLCFAs, the availability of such genes would offer the potential to modify the wax load itself. This offers the potential to modify the susceptibility of plants to environmental stresses such as ultraviolet light, heat and drought, as well as the ability of plants to withstand insects and pathogens. The present invention is directed towards nucleic acids that encode  
 30 condensing enzymes for VLCFA synthesis.

#### Summary of the Invention

The present invention provides nucleic acids (cDNAs and genomic clones) that encode a key enzyme in the synthesis of VLCFAs in plant epidermal cells. The activity of this enzyme is referred to as very long chain fatty acid elongase; the activity is required for synthesis of VLCFAs of greater than 24 carbons in length. It is shown that co-suppression of the *CUT1* gene in plants can disrupt VLCFA synthesis which results in plants having none of the protective wax usually found on stem surfaces. In addition, it is shown that such plants are conditionally male sterile: when grown under normal humidity, the plants are male sterile, but fertility can be restored by growth in an elevated humidity environment.

defined earlier  
as 20 or more  
carbons long

The invention thus provides the *CUT1* cDNA and gene nucleotide sequences ("*CUT1* nucleic acids") and the amino acid sequence of the *CUT1* protein. In one embodiment, the *CUT1* nucleic acids disclosed are from *Arabidopsis thaliana*. The open reading frame of the *Arabidopsis CUT1* cDNA molecule encodes an enzyme of 497 amino acids which catalyzes the addition of 2C units to pre-existing

5 C24 or longer fatty acids.

Also encompassed within the scope of this invention are transformation vectors that include at least a portion of the *CUT1* nucleic acid molecules. Such vectors may be transformed into plants to produce transgenic plants with modified VLCFA compositions (relative to non-transgenic plants of the same species). Depending on the particular sequences incorporated into the vector, transformation with

10 the *CUT1* cDNA, gene or derivatives thereof can be used to modify agronomically important traits, including the presence, composition and thickness of epicuticular wax layers on leaves and stems, seed coat fatty acids, seed oil composition and male sterility. Typically, such vectors include regulatory sequences, such as promoters, operably linked to the *CUT1* open reading frame or a derivative of the *CUT1* nucleic acids. For example, VLCFA synthesis may be altered by introducing into a plant a

15 transformation vector that includes a sense or antisense version of the *CUT1* cDNA. Transgenic plants having modified VLCFA compositions and which are transformed with such recombinant transformation vectors are also provided by this invention.

In one aspect of the invention, transformation with sense or antisense versions of the *CUT1* nucleic acids may be used to produce plants having modified epicuticular wax layers on the aerial parts

20 of the plants, such as the leaves and stems. A modified epicuticular wax layer may be modified in physical respects, such as thickness of the wax layer, or in composition. Because these layers play a role in the ability of plants to resist environmental stresses, such as drought and ultraviolet light, as well as insects and pathogens, transformation with vectors including forms of the *CUT1* nucleic acids may be used to produce plants with particular agronomic advantages. Producing plants with modified

25 epicuticular wax composition may be achieved by introducing into the plants a vector in which the *CUT1* nucleic acid (or a derivative thereof) is operably linked to a promoter that directs expression of the open reading frame in the epidermal cells. The CaMV 35S promoter and the endogenous *CUT1* gene promoter are examples of regulatory sequences that may be suitable for this purpose.

Agronomically important traits in addition to wax composition may also be modified using the

30 *CUT1* nucleic acids of the present invention. For example, the fatty acid composition of the seed coat and the fatty acid composition of seed oil may be modified by transforming plants with the *CUT1* cDNA or derivatives thereof. Preferably, where it is desired to modify aspects of seed VLCFA composition, the introduced *CUT1* nucleic acid sequence will be operably linked to a promoter known to direct expression in seed tissues. Seed-specific promoters include the napin promoter of *Brassica napus* (Lee et al., 1991). In addition, transformation with the *CUT1* nucleic acids or derivatives thereof may be

35 used to disrupt VLCFA synthesis in pollen, resulting in conditionally male sterile plants. Such plants are useful in plant breeding programs.

While the invention provides *CUT1*-encoding nucleic acids from *Arabidopsis*, it additionally

encompasses homologs, orthologs and variants and derivatives of these sequences, as well as homologs, orthologs and variants of the CUT1 polypeptide sequence. Thus, in one aspect of the invention, nucleic acid molecules that comprise specified regions of these sequences are provided. Exemplary of such nucleic acid molecules are oligonucleotides that are useful as probes or primers to detect and amplify *CUT1*-  
5 encoding nucleic acids from other plant species. Such oligonucleotides are useful as hybridization probes or PCR primers, and typically comprise at least 15 consecutive bases of the disclosed *CUT1* nucleic acid sequences. In other embodiments, such oligonucleotides comprise longer regions of the disclosed *CUT1* sequences, such as at least 20, 25 or 30 consecutive nucleotides.

In another aspect, the invention provides compositions and methods for isolating nucleic acid  
10 sequences that encode enzymes having CUT1 activity from other plant species. Typically, such methods involve hybridizing probes or primers derived from the disclosed *Arabidopsis* sequences to nucleic acids obtained or derived from such other plant species.

Homologous and orthologous sequences to *Arabidopsis CUT1* nucleic acid and CUT1 amino acid sequences share key functional and structural characteristics with the disclosed *Arabidopsis* sequences.  
15 Functionally, such sequences encode (or comprise) a polypeptide that catalyzes the very long chain fatty acid elongation as described above. Structurally, such sequences share a specified structural relationship with the disclosed sequences. By way of example, in certain embodiments, homologous amino acid sequences have at least 70% sequence identity with the *Arabidopsis CUT1* amino acid sequence. In other embodiments, homologous nucleic acid sequences hybridize under stringent conditions to the disclosed  
20 *Arabidopsis CUT1* nucleic acid sequences.

Another aspect of the invention relates to the purified CUT1 enzyme itself. Having provided nucleic acid molecules that encode this enzyme, the invention also facilitates the expression of CUT1 enzyme in heterologous systems, including *E. coli*, yeast and baculovirus expression systems. Thus, the invention permits the large scale production of the enzyme for agricultural and other applications.

25 In another aspect of the invention the promoter sequence of the *CUT1* gene is disclosed. This promoter sequence confers epidermis-specific expression, and may be used to express a variety of nucleic acids in an epidermis-specific manner.

### Detailed Description of the Invention

#### 30 I. Definitions

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V* published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew et al (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.),  
35 *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8). The nomenclature for DNA bases as set forth at 37 CFR § 1.822 and the standard three letter codes for amino acid residues are used herein.

In order to facilitate review of the various embodiments of the invention, the following

definitions of terms are provided:

**CUT1 protein:** The defining functional characteristic of a CUT1 protein is its enzymatic activity, specifically its very long chain fatty acid elongase activity. This activity is manifested as the catalysis of one or more steps in the addition of 2 carbon moieties (such as malonyl-coenzyme A) to pre-existing very long chain fatty acids (VLCFAs). In a preferred embodiment, a CUT1 protein catalyzes one or more steps in the addition of 2 carbon moieties to pre-existing long chain fatty acids of at least 24 carbon units in length. This activity can be measured by the assay described below.

This invention provides a cDNA and a gene encoding a CUT1 enzyme from *Arabidopsis thaliana*. However the invention is not limited to this particular CUT1 protein: other nucleotide sequences which encode CUT1 proteins are also part of the invention, including variants on the disclosed *Arabidopsis* cDNA and gene sequences and orthologous sequences from other plant species, including naturally occurring variants, such as sequences from other ecotypes, species and natural polymorphisms, the cloning of which is now enabled. Such sequences share the essential functional characteristic of encoding an enzyme having very long chain fatty acid elongase activity. Nucleic acid sequences that encode CUT1 proteins and the proteins encoded by such nucleic acids share not only this functional characteristic, but also a specified level of sequence similarity (or sequence identity), as addressed below. The concept of sequence identity can also be expressed in the ability of two sequences to hybridize to each other under stringent conditions.

**Sequence identity:** the similarity between two nucleic acid sequences, or two amino acid sequences is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are.

Methods of alignment of sequences for comparison are well-known in the art. Various programs and alignment algorithms are described in: Smith and Waterman (1981); Needleman and Wunsch (1970); Pearson and Lipman (1988); Higgins and Sharp (1988); Higgins and Sharp (1989); Corpet et al. (1988); and Pearson et al. (1994). Altschul et al. (1994) presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) is available from several sources, including the National Center for Biological Information (NCBI, Bethesda, MD) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. It can be accessed at <http://www.ncbi.nlm.nih.gov/BLAST/>. A description of how to determine sequence identity using this program is available at [http://www.ncbi.nlm.nih.gov/BLAST/blast\\_help.html](http://www.ncbi.nlm.nih.gov/BLAST/blast_help.html).

Homologs of the *Arabidopsis* CUT1 protein are characterized by possession of at least 70% sequence identity counted over the full length alignment with the disclosed *Arabidopsis* CUT1 amino acid sequence using the NCBI Blast 2.0, gapped blastp set to default parameters. Such homologous peptides will more preferably possess at least 75%, more preferably at least 80% and still more preferably at least 90% or 95% sequence identity with the *Arabidopsis* CUT1 amino acid sequence determined by this method. When less than the entire sequence is being compared for sequence identity, homologs will



possess at least 75% and more preferably at least 85% and more preferably still at least 90% or 95% sequence identity over short windows of 10-20 amino acids. Methods for determining sequence identity over such short windows are described at [http://www.ncbi.nlm.nih.gov/BLAST/blast\\_FAQs.html](http://www.ncbi.nlm.nih.gov/BLAST/blast_FAQs.html).

Homologs having the sequence identities described above will, in some embodiments, also possess

- 5 VLCFA elongase activity. One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is entirely possible that strongly significant homologs could be obtained that fall outside of the ranges provided. The present invention provides not only the peptide homologs are described above, but also nucleic acid molecules that encode such homologs.

- Homologs of the *Arabidopsis CUT1* cDNA and gene are similarly characterized by possession of  
10 at least 60% sequence identity counted over the full length alignment with the disclosed *Arabidopsis* cDNA or gene sequence using the NCBI Blast 2.0, gapped blastn set to default parameters. Such homologous nucleic acids will more preferably possess at least 70%, more preferably at least 80% and still more preferably at least 90% or 95% sequence identity determined by this method. When less than the entire sequence is being compared for sequence identity, homologs will possess at least 85% and more  
15 preferably at least 90% and more preferably still at least 95% sequence identity over 30 nucleotide windows. Homologs having the sequence identities described above will, in some embodiments, also encode a polypeptide having VLCFA elongase activity. However, homologs as defined above are useful for modifying VLCFA elongase activity in transgenic plants (for example, as used in antisense constructs) even when they do not encode a functional peptide. Again, one of skill in the art will appreciate that these  
20 sequence identity ranges are provided for guidance only; it is entirely possible that strongly significant nucleic acid homologs could be obtained that fall outside of the ranges provided.

- Another indication that two nucleic acid molecules are substantially homologous is that the two molecules hybridize to each other under stringent conditions when one molecule is used as a hybridization probe, and the other is present in a biological sample, e.g., genomic material from a cell. Specific  
25 hybridization means that the molecules hybridize substantially only to each other and not to other molecules that may be present in the genomic material. Stringent conditions are sequence dependent and are different under different environmental parameters. Generally, stringent conditions are selected to be about 5°C to 20°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength and pH. The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of the  
30 target sequence hybridizes to a perfectly matched probe. Conditions for nucleic acid hybridization and calculation of stringencies can be found in Sambrook et al. (1989) and Tijssen (1993). Hybridization conditions and stringencies are further discussed below.

- Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. It is understood that changes in  
35 nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequence that all encode substantially the same protein.

**Probes and primers:** Nucleic acid probes and primers may readily be prepared based on the nucleic acids provided by this invention. A probe comprises an isolated nucleic acid attached to a

detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, e.g., in Sambrook et al. (1989) and Ausubel et al. (1987).

5 Primers are short nucleic acids, preferably DNA oligonucleotides 15 nucleotides or more in length. Primers may be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art.

10 Methods for preparing and using probes and primers are described, for example, in Sambrook et al. (1989), Ausubel et al. (1987), and Innis et al., (1990). PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA). One of skill in the art will appreciate that the specificity of a particular probe or primer increases with its length. Thus, for  
15 example, a primer comprising 20 consecutive nucleotides of the *Arabidopsis CUT1* cDNA or gene will anneal to a target sequence (e.g., a corresponding CUT1 gene from *Zea mays*) with a higher specificity than a corresponding primer of only 15 nucleotides. Thus, in order to obtain greater specificity, probes and primers may be selected that comprise 20, 25, 30, 35, 40, 50 or more consecutive nucleotides of the *Arabidopsis CUT1* cDNA or gene sequences. Such probes and primers are useful for obtaining CUT1  
20 nucleic acid molecules (cDNA, genomic sequences, and portions of these molecules) both from *Arabidopsis* and other plant species.

**Vector:** A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in the host cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic  
25 elements known in the art.

**Transformed:** A transformed cell is a cell into which has been introduced a nucleic acid molecule by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transformation with *Agrobacterium* vectors, transfection with viral vectors, transformation with plasmid  
30 vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

**Isolated:** An "isolated" biological component (such as a nucleic acid or protein) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids and proteins which have been "isolated" thus include nucleic acids and  
35 proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

**Purified:** The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified CUT1 protein preparation is one in which the CUT1 protein is more

enriched than the protein is in its natural environment within a cell. Preferably, a preparation of CUT1 protein is purified such that CUT1 protein represents at least 50% of the total protein content of the preparation.

5 **Operably linked:** A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter effects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

10 **Recombinant:** A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

15 **Ortholog:** two nucleotide or amino acid sequences are orthologs of each other if they share a common ancestral sequence and diverged when a species carrying that ancestral sequence split into two species. Orthologous sequences are also homologous sequences.

20 **Transgenic plant:** as used herein, this term refers to a plant that contains recombinant genetic material not normally found in plants of this type and which has been introduced into the plant in question (or into progenitors of the plant) by human manipulation. Thus, a plant that is grown from a plant cell into which recombinant DNA is introduced by transformation is a transgenic plant, as are all offspring of that plant which contain the introduced DNA (whether produced sexually or asexually).

## II. Sequence Listing and Figures

25 The nucleic and amino acid sequences listed in the accompanying sequence listing are showed using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand.

Seq. I.D. No. 1 shows the nucleotide sequence of the *CUT1* gene and the encoded amino acid sequence.

30 Seq. I.D. No. 2 shows the nucleotide sequence of the *CUT1* cDNA.

Seq. I.D. No. 3 shows the nucleotide sequence of the *CUT1* open reading frame.

Seq. I.D. No. 4 shows the amino acid sequence of the CUT1 protein.

Seq. I.D. Nos. 5 – 11 show primers useful in PCR amplification of various regions of the *CUT1* gene, cDNA or ORF.

35 Seq. I.D. No.12 shows the promoter region of the *CUT1* genomic clone.

Fig. 1 shows the pathways of wax biosynthesis in *Arabidopsis*.

### III. Isolation and Characterization of the *CUT1* cDNA

5 The *CUT1* cDNA was initially identified using a TBLASTN homology search (Altschul et al., 1990) of the database of expressed sequenced tags (ESTs) of anonymous *Arabidopsis* cDNA clones (Newman et al., 1994) using the deduced amino acid sequence of the *FAE1* gene. The search found 14 ESTs in the database which had open reading frames with significant homology to *FAE1*. These ESTs did not correspond to known condensing enzymes such as chalcone synthase or 3-ketoacyl-acyl carrier  
10 protein synthase III.

One of these ESTs was selected for further investigation, and the corresponding full length cDNA was isolated. This cDNA is herein referred to as the *CUT1* cDNA. Sequencing demonstrated that the *CUT1* cDNA was 1829 nucleotides long, approximately the size of the *FAE1* transcript (James et al., 1995). The *CUT1* cDNA contains one open reading frame of 497 amino acids, which is shorter than  
15 both the *FAE1* sequence (506 amino acids) and the jojoba KCS (521 amino acids). The *CUT1* cDNA and the protein it encodes are shown in Seq. I.D. Nos. 2 and 4, respectively.

There is an in frame stop codon, TAA, 15 nucleotides upstream of the most 5' ATG, suggesting that this sequence indeed represents the full length amino acid sequence of the protein. Thus, the *CUT1* cDNA as depicted in Seq. I.D. No. 2 has a 5' untranslated region of 58 nucleotides, an open reading  
20 frame of 1491 nucleotides and a 3' untranslated region of 258 nucleotides, excluding the poly(A) tail (22 As). Comparison of the deduced amino acid sequence of the *CUT1* protein to *FAE1* revealed that they are 50.0% identical and 74.7% similar.

### IV. Isolation and Characterization of the *CUT1* Gene

25 An *Arabidopsis* *CUT1* genomic clone was isolated from a genomic library in  $\lambda$ GEM11 by probing nitrocellulose plaque lifts with a full-length *CUT1* cDNA clone. A 2.5 kb long *SalI* fragment containing 580 bp of the coding sequence and 1951 bp of the 5' upstream region was subcloned into the *SalI* site of pT7T3 18U plasmid (Pharmacia), followed by complete sequencing on both strands. The sequence of this genomic clone is shown in Seq. I.D. No. 1.

30 *In situ* hybridization studies in developing shoots, leaves and siliques of *Arabidopsis* indicated epidermis-specific expression of the *CUT1* gene, as expected of a gene encoding an enzyme involved in wax biosynthesis.

### V. Analysis of the *CUT1* Promoter

35 In order to confirm the tissue and cell specificity of the *CUT1* promoter, 5' flanking sequences from the *CUT1* genomic clone were operably linked to the *uidA* reporter gene encoding  $\beta$ -glucuronidase (GUS). Two constructs were made, one having a 1.9 kb promoter fragment and the second containing a truncated 1.2 kb promoter. These promoter-GUS fusions were introduced into *Arabidopsis* and tobacco by *Agrobacterium*-mediated transformation and the promoter function characterized in transgenic plants.

40 To obtain the 1.9 and 1.2 kb regions of the *CUT1* promoter sequence, synthetic oligonucleotides

homologous to portions of the 5' untranslated region of the genomic clone were used as primers to amplify either a 1949 bp or a 1209 bp promoter fragment by PCR. As shown in Figure 1, the upstream primer was 5'-GTGCTTTATATATGTTTG-3' (cutpro3) (Seq. I.D. No. 5) in combination with the downstream primer 5'-CGTCGGAGAGTTTAAATG-3' (cutpro1) (Seq. I.D. No. 6) for the PCR-synthesis of the 1949 bp fragment, and 5'-CTTCGATATCGGTTGTTG-3' (cutpro2) (Seq. I.D. No. 7) and cutpro1 for the amplification of the 1209 bp fragment. In both cases, the amplified products were subcloned in the *HincII* site of the plasmid pT7T318U (Pharmacia). The inserts were then cleaved out with *HindIII* and *XbaI* and directionally subcloned into the corresponding sites of the binary Ti plasmid pBI101 (Clontech), which contains a promoterless GUS gene (Jefferson et al. 1987). The *pCUT1-GUS* fusion constructs in pBI101 were introduced into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) by electroporation and selected for resistance to kanamycin (50 µg/ml).

For transformation of tobacco, *Agrobacterium* harbouring the *pCUT1-GUS* construct was co-cultivated with leaf pieces of *Nicotiana tabacum* SR1 and transformants were selected with kanamycin (100mg/mL) on solid medium (Lee and Douglas, 1996). *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia was transformed with *pCUT1-GUS* binary vector using a combination of *in planta* (Chang et al., 1994, Katavic et al., 1994) and vacuum infiltration methods (Bechtold et al., 1993). Plants were grown until the primary inflorescence shoots reached 1-2 cm in height, when this bolts were cut off. The wound site was inoculated with 50 mL of an overnight *Agrobacterium* culture. After 4-6 days a number of secondary inflorescences that appeared were cut off, and vacuum infiltration was performed on these plants using the conditions described by Bechtold et al. (1993). Screening for transformed seed was done on 50µg/mL kanamycin as described previously (Katavic et al., 1994).

Tissue sections of transgenic plants containing the *pCUT1-GUS* constructs were placed in 100 mM NaPO<sub>4</sub> (pH7) and 1 mM spermidine for 15 min, then incubated at 37° C in 0.5 K<sub>3</sub>[Fe(CN)<sub>6</sub>], 0.01 % Triton X-100, 1mM EDTA, 10 mM β-mercaptoethanol, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM NaPO<sub>4</sub> (pH7), until a blue color appeared (after approximately 1 hr). Following incubation with the substrate, chlorophyll was removed from the sections using a graded ethanol series.

In both recipient plant species, *Arabidopsis* and tobacco, *CUT1* expression pattern mirrored that observed in the *in situ* experiments. Furthermore, both long and short *CUT1* promoter fragments targeted expression of the *uidA* gene exclusively to the epidermis. No GUS expression was detected in any of the other cell types in the stems or leaves of transgenic plants. Thus, the *Arabidopsis CUT1* promoter is regulated in a tissue specific, and cell specific manner, and epidermis specificity appears to be retained even in unrelated plant species like tobacco. In addition, no differences in the strength of expression were detected between the 1.9 kb and 1.2 kb promoter.

## VI. Preferred Methods for Producing *CUT1* Nucleic Acids

With the provision of the *CUT1* cDNA and gene (the "*CUT1* nucleic acids") the polymerase chain reaction (PCR) may now be utilized in a preferred method for producing the *CUT1* nucleic acids. PCR amplification of the *CUT1* cDNA sequence may be accomplished either by direct PCR from a plant

cDNA library or by Reverse-Transcription PCR (RT-PCR) using RNA extracted from plant cells as a template. Methods and conditions for both direct PCR and RT-PCR are known in the art and are described in Innis et al. (1990). Suitable plant cDNA libraries for direct PCR include the *Arabidopsis* cDNA library described by Newman et al. (1994). Similarly, the *CUT1* genomic sequence may be amplified directly from genomic DNA extracted from plants, or from plant genomic DNA libraries. Amplification may be used to obtain the full length cDNA or genomic sequence, or may be used to amplify selected portions of these molecules (for example for use in antisense constructs)

The selection of PCR primers will be made according to the portions of the *CUT1* nucleic acids which are to be amplified. Variations in amplification conditions may be required to accommodate primers of differing lengths; such considerations are well known in the art and are discussed in Innis et al. (1990), Sambrook et al. (1989), and Ausubel et al (1987). By way of example only, the entire *CUT1* cDNA molecule as shown in Seq. I.D. No. 2 may be amplified using the following combination of primers:

primer 1 5' AAATACCCTAATCACATTTTGTA 3' (Seq. I.D. No. 8)  
 primer 2 5' TTAAACAGAGAGAAATATTCTTA 3' (Seq. I.D. No. 9)

The open reading frame portion of the cDNA may be amplified using the following primer pair:  
 primer 3 5' ATGCCTCAGGCACCGATGCCAGAG 3' (Seq. I.D. No. 10)  
 primer 4 5' CAGCACGAGAACTAAAAAATACC 3' (Seq. I.D. No. 11)

These primers are illustrative only; it will be appreciated by one skilled in the art that many different primers may be derived from the provided sequences in order to amplify particular regions of the *CUT1* sequences. Resequencing of PCR products obtained by these amplification procedures is recommended; this will facilitate confirmation of the amplified *CUT1* sequence and will also provide information on natural variation on this sequence in different ecotypes and plant populations.

Oligonucleotides which are derived from the *CUT1* nucleic acid sequences and which are suitable for use as PCR primers to amplify the *CUT1* nucleic acid sequences are encompassed within the scope of the present invention. Preferably, such oligonucleotide primers will comprise a sequence of 15-20 consecutive nucleotides of the *CUT1* nucleic acid sequences. To enhance amplification specificity, primers comprising at least 20-30 consecutive nucleotides of these sequences may also be used.

30

#### VII. Cloning *CUT1* Variants

With the provision herein of the *CUT1* nucleic acid sequences, the cloning by standard methodologies of corresponding cDNAs and genes from other ecotypes and plant species, as well as polymorphic forms of the disclosed sequences is now enabled. Thus, the present invention includes methods of isolating a nucleotide sequence encoding a plant very long chain fatty acid elongation enzyme from a plant. Both conventional hybridization and PCR amplification procedures may be utilized to clone such sequences. Common to both of these techniques is the hybridization of probes or primers derived from the disclosed *CUT1* nucleic acid sequences to a target nucleotide preparation, which may

be, in the case of conventional hybridization approaches, a cDNA or genomic library or, in the in the case of PCR amplification, extracted genomic DNA, mRNA, a cDNA library or a genomic library.

Direct PCR amplification may be performed on cDNA libraries prepared from the plant species in question, or RT-PCR may be performed using mRNA extracted from the plant cells using standard methods. PCR primers will comprise at least 15 consecutive nucleotides of the *CUT1* nucleic acid sequences. One of skill in the art will appreciate that sequence differences between the disclosed *CUT1* nucleic acid sequences and the target gene to be amplified may result in lower amplification efficiencies. To compensate for this, longer PCR primers or lower annealing temperatures may be used during the amplification cycle. Where lower annealing temperatures are used, sequential rounds of amplification using nested primer pairs may be necessary to enhance specificity.

For conventional hybridization techniques, the hybridization probe is preferably labeled with a detectable label such as a radioactive label, and the probe is of at least 20 nucleotides in length. As is well known in the art, increasing length of hybridization probes tends to give enhanced specificity. The labeled probe derived from, for example, the *CUT1* cDNA sequence may be hybridized to a plant cDNA or genomic library and the hybridization signal detected using means known in the art. The hybridizing colony or plaque (depending on the type of library used) is then purified and the cloned sequence contained in that colony or plaque isolated and characterized.

#### VIII. Use of the *CUT1* Nucleic Acids to Produce Plants with Modified VLCFA Composition

Once a gene or cDNA ("nucleic acid") encoding a protein involved in the determination of a particular plant characteristic has been isolated, standard techniques may be used to express the nucleic acid in transgenic plants in order to modify that particular plant characteristic. The basic approach is to clone the nucleic acid into a transformation vector, such that it is operably linked to control sequences (e.g., a promoter) which direct expression of the open reading frame in plant cells. The transformation vector is then introduced into plant cells by one of a number of techniques (e.g., electroporation) and progeny plants containing the introduced nucleic acid are selected. Preferably all or part of the transformation vector will stably integrate into the genome of the plant cell. That part of the transformation vector which integrates into the plant cell and which contains the introduced nucleic acid and associated sequences for controlling expression (the introduced "transgene") may be referred to as the recombinant expression cassette.

Selection of progeny plants containing the introduced transgene may be made based upon the detection of an altered phenotype. Such a phenotype may result directly from the nucleic acid cloned into the transformation vector or may be manifested as enhanced resistance to a chemical agent (such as an antibiotic) as a result of the inclusion of a dominant selectable marker gene incorporated into the transformation vector.

The choice of (a) control sequences and (b) how the nucleic acid (or selected portions of the nucleic acid) are arranged in the transformation vector relative to the control sequences determine, in

part, how the plant characteristic affected by the introduced nucleic acid is modified. For example, the control sequences may be tissue specific, such that the nucleic acid is only expressed in particular tissues of the plant (e.g., pollen) and so the affected characteristic will be modified only in those tissues. The nucleic acid sequence may be arranged relative to the control sequence such that the nucleic acid transcript is expressed normally, or in an antisense orientation. Expression of an antisense RNA corresponding to the cloned nucleic acid will result in a reduction of the targeted gene product (the targeted gene product being the protein encoded by the plant gene from which the introduced nucleic acid was derived). Over-expression of the introduced nucleic acid, resulting from a plus-sense orientation of the nucleic acid relative to the control sequences in the vector, may lead to an increase in the level of the gene product, or may result in co-suppression (also termed "sense suppression") of that gene product.

Successful examples of the modification of plant characteristics by transformation with cloned nucleic acid sequences are replete in the technical and scientific literature. Selected examples, which serve to illustrate the current knowledge in this field of technology, and which are herein incorporated by reference, include:

U.S. Patent No. 5,451,514 to Boudet (modification of lignin synthesis using antisense RNA and co-suppression);

U.S. Patent No. 5,443,974 to Hitz (modification of saturated and unsaturated fatty acid levels using antisense RNA and co-suppression);

U.S. Patent No. 5,530,192 to Murase (modification of amino acid and fatty acid composition using antisense RNA);

U.S. Patent No. 5,455,167 to Voelker (modification of medium chain fatty acids)

U.S. Patent No. 5,231,020 to Jorgensen (modification of flavonoids using co-suppression);

U.S. Patent No. 5,583,021 to Dougherty (modification of virus resistance by expression of plus-sense untranslatable RNA);

WO 96/13582 (modification of seed VLCFA composition using over expression, co-suppression and antisense RNA in conjunction with the *Arabidopsis* FAE1 gene); and

WO 95/15387 (modification of seed VLCFA composition using over expression of jojoba wax synthesis gene).

These examples include descriptions of transformation vector selection, transformation techniques and the construction of constructs designed to over-express the introduced nucleic acid or to express antisense RNA corresponding to the nucleic acid. In light of the foregoing and the provision herein of the *CUT1* nucleic acids, it is thus apparent that one of skill in the art will be able to introduce these nucleic acids, or derivative forms of these molecules (e.g., antisense forms), into plants in order to produce plants having modified VLCFA compositions. Examples one and two below provides illustrations of this in which the *CUT1* cDNA is operably linked to the CaMV 35S promoter sequence, cloned into the pBIN19 transformation vector and introduced into *Arabidopsis* using a vacuum infiltration method.



As reported in Example one, certain of the plants transformed in this way had no detectable epicuticular wax layers, indicating that transformation with the *CUT1* cDNA had disrupted normal VLCFA synthesis in the plant epidermal cells. Such disruption is likely attributable to the phenomenon termed co-suppression (or sense-suppression). These plants are thus referred to as "*CUT1*-suppressed".

- 5 This phenomenon may be affected by factors such as positional location of the introduced sequences in the plant genome.

Over-expression of CUT1 protein in transgenic plants, resulting in plants enhanced epicuticular wax layers will be a useful agronomic trait, providing increased drought and insect resistance. For example, drought resistance in rice is associated with high wax lines rich in C<sub>29</sub>, C<sub>31</sub> and C<sub>33</sub> alkanes  
10 (O'Toole and Cruz, 1983; Haque et al., 1992). Increased wax deposition in transgenic plants can be accomplished by overexpression of CUT1 protein, while the identification of the *CUT1* promoter allows targeting of lipid modification enzymes such as desaturases, thioesterases and other condensing enzymes with different specificities to the epidermal cells to modify wax composition.

Transformation of plants with the *CUT1* nucleic acids or derivatives thereof may be used to  
15 modify other plant characteristics, such as seed coat composition and seed oil composition. Because condensing enzymes are pivotal enzymes in the synthesis of VLCFAs, controlling levels of accumulation of VLCFAs and their acyl chain length (Millar and Kunst, 1997) through the manipulation of CUT1 expression will permit the production of plants having novel fatty acid compositions. For instance, the accumulation of VLCFAs in tobacco seed expressing *FAEI* from *Arabidopsis* (Millar and Kunst, 1997)  
20 raises the possibility of producing VLCFAs in plant species that currently do not synthesize VLCFAs. In addition, targeting of CUT1 to seeds will be useful to produce crop plants capable of synthesising new, agronomically important VLCFAs in seed oil.

Disruption of CUT1 activity in transgenic plants also provides a simple means for obtaining conditional male sterility in plants (see Example two). One of the major factors contributing to increases  
25 in crop productivity is the development of hybrid varieties of crops. Several different breeding strategies have been used to produce hybrid seed, but none of these strategies can be used as a general approach in all crop plants (Goldberg et al., 1993). As an alternative, genetically engineered systems and strategies for male fertility control that are applicable to a wide range of crops have recently been developed. For example, nuclear male sterility has been engineered by (1) tapetum-specific expression of a bacterial  
30 RNase gene (Mariani et al., 1990, 1992), (2) overexpression of the *rolC* gene from *Agrobacterium rhizogenes* (Fladung, 1990; Schmülling et al., 1988, 1992), (3) expression of glucanase that disrupts the callose wall of the microsporophyte prematurely (Tsuchiya et al., 1995; Worrall et al., 1992), (4) the inhibition of flavonoid biosynthetic genes like chalcone synthase and dihydroflavonol 4-reductase (van der Krol et al., 1988, 1990; van der Meer et al., 1992; Napoli et al., 1990; Taylor and Jorgensen, 1992), and (5)  
35 altered expression of stilbene synthase (Fischer et al., 1997). However, in most of these cases the restoration of fertility is not simple, or not easily controlled. In contrast, conditional male sterility caused by suppression of CUT1 activity is easily reversible under high relative humidity.

The selection of vectors and promoters appropriate for targeting particular characteristics for

modification (such as seed-specific expression) are well known; the following paragraphs set forth general guidance on the various options available in producing transgenic plants having modified VLCFA composition.

5           a.       Plant Types

VLCFAs are found in all plant types, and thus DNA molecules according to the present invention (e.g., the *CUT1* cDNA, gene, homologs and antisense forms thereof) may be introduced into any plant type in order to modify the VLCFA composition of the plant. Thus, the sequences of the present invention may be used to modify VLCFA composition in any higher plant, including  
10   monocotyledonous and dicotyledonous plants, including, but not limited to maize, wheat, rice, barley, soybean, beans in general, rape/canola, alfalfa, flax, sunflower, safflower, brassica, cotton, flax, peanut, clover; vegetables such as lettuce, tomato, cucurbits, potato, carrot, radish, pea, lentils, cabbage, broccoli, brussel sprouts, peppers; tree fruits such as apples, pears, peaches, apricots; flowers such as carnations and roses.

15

          b.       Vector Construction, Choice of Promoters

A number of recombinant vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described including those described in Pouwels et al., (1987), Weissbach and Weissbach, (1989), and Gelvin et al., (1990). Typically, plant transformation  
20   vectors include one or more cloned plant genes (or cDNAs) under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant transformation vectors typically also contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally-or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site,  
25   and/or a polyadenylation signal.

Examples of constitutive plant promoters which may be useful for expressing *CUT1* nucleic acids include: the cauliflower mosaic virus (CaMV) 35S promoter, which confers constitutive, high-level expression in most plant tissues (see, e.g., Odel et al., 1985, Dekeyser et al., 1990, Terada and Shimamoto, 1990); the nopaline synthase promoter (An et al., 1988); and the octopine synthase promoter  
30   (Fromm et al., 1989).

A variety of plant gene promoters that are regulated in response to environmental, hormonal, chemical, and/or developmental signals, also can be used for expression of *CUT1* nucleic acids in plant cells, including promoters regulated by: (a) heat (Callis et al., 1988); (b) light (e.g., the pea *rbcS-3A* promoter, Kuhlmeier et al., 1989, the maize *rbcS* promoter, Schaffner and Sheen, 1991, and the  
35   chlorophyll a/b binding protein promoter, Simpson et al., 1985); (c) hormones, such as abscisic acid (Marcotte et al., 1989); (d) wounding (e.g., *wun1*, Siebertz et al., 1989); and (e) chemicals such as methyl jasmonate or salicylic acid. It may also be advantageous to employ tissue-specific promoters, such as those described by Roshal et al., (1987), Scherthaner et al., (1988), and Bustos et al., (1989).

Alternatively, tissue specific (root, leaf, flower, and seed for example) promoters (Carpenter et al. 1992, Denis et al. 1993, Opperman et al. 1993, Stockhouse et al. 1997; Roshal et al., 1987; Schernthaner et al., 1988; and Bustos et al., 1989) can be fused to the coding sequence to obtain particular expression in respective organs. In addition, the timing of the expression can be controlled by using promoters such as those acting at senescencing (Gan and Amasino 1995) or late seed development (Odell et al. 1994). The promoter region of the *CUT1* genomic sequence disclosed herein confers epidermis-specific expression in *Arabidopsis* and tobacco. Accordingly, the native promoter may be used to obtain epidermis-specific expression of the introduced transgene.

For producing conditionally male sterile plants by blocking *CUT1* activity in pollen, it is preferable to use a pollen-specific promoter (so as to avoid pleiotropic effects). Thus, the *CUT1* coding region may be expressed under the control of the tapetum-specific promoters such as TA29 (Mariani et al., 1990, 1992), *MS2* (Aarts et al., 1997), and *tap1* (Nacken et al., 1991).

Plant transformation vectors may also include RNA processing signals, for example, introns, which may be positioned upstream or downstream of the *CUT1* nucleic acid sequence in the transgene.

In addition, the expression vectors may also include additional regulatory sequences from the 3'-untranslated region of plant genes, e.g., a 3' terminator region to increase mRNA stability of the mRNA, such as the PI-II terminator region of potato or the octopine or nopaline synthase 3' terminator regions.

Finally, as noted above, plant transformation vectors may also include dominant selectable marker genes to allow for the ready selection of transformants. Such genes include those encoding antibiotic resistance genes (e.g., resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin) and herbicide resistance genes (e.g., phosphinothricin acetyltransferase).

#### c. Arrangement of *CUT1* Nucleic Acids in Vector

As noted above, the particular arrangement of the *CUT1* nucleic acid in the transformation vector will be selected according to the expression of the nucleic acid desired.

Where enhanced VLCFA synthesis is desired, the *CUT1* nucleic acid may be operably linked to a constitutive high-level promoter such as the CaMV 35S promoter. Modification of VLCFA synthesis may also be achieved by introducing into a plant a transformation vector containing a variant form of the *CUT1* nucleic acid, for example a form which varies from the exact nucleotide sequence of the *CUT1* nucleic acid, but which encodes a protein that retains the functional characteristic of the *CUT1* protein, i.e., very long chain fatty acid elongation activity.

In contrast, a reduction of VLCFA synthesis may be obtained by introducing antisense constructs based on the *CUT1* nucleic acid sequence into plants. For antisense suppression, the *CUT1* nucleic acid is arranged in reverse orientation relative to the promoter sequence in the transformation vector. The introduced sequence need not be the full length *CUT1* nucleic acid, and need not be exactly homologous to the *CUT1* nucleic acid. Generally, however, where the introduced sequence is of shorter length, a higher degree of homology to the native *CUT1* sequence will be needed for effective antisense

suppression. Preferably, the introduced antisense sequence in the vector will be at least 30 nucleotides in length, and improved antisense suppression will typically be observed as the length of the antisense sequence increases. Preferably, the length of the antisense sequence in the vector will be greater than 100 nucleotides. Transcription of an antisense construct as described results in the production of RNA molecules that are the reverse complement of mRNA molecules transcribed from the endogenous *CUT1* gene in the plant cell. Although the exact mechanism by which antisense RNA molecules interfere with gene expression has not been elucidated, it is believed that antisense RNA molecules bind to the endogenous mRNA molecules and thereby inhibit translation of the endogenous mRNA.

Suppression of endogenous *CUT1* gene expression can also be achieved using ribozymes.

Ribozymes are synthetic RNA molecules that possess highly specific endoribonuclease activity. The production and use of ribozymes are disclosed in U.S. Patent No. 4,987,071 to Cech and U.S. Patent No. 5,543,508 to Haselhoff, which are hereby incorporated by reference. The inclusion of ribozyme sequences within antisense RNAs may be used to confer RNA cleaving activity on the antisense RNA, such that endogenous mRNA molecules that bind to the antisense RNA are cleaved, which in turn leads to an enhanced antisense inhibition of endogenous gene expression.

Constructs in which the *CUT1* nucleic acid (or variants thereon) are over-expressed may also be used to obtain co-suppression of the endogenous *CUT1* gene in the manner described in U.S. Patent No. 5,231,021 to Jorgensen. Such co-suppression (also termed sense suppression) does not require that the entire *CUT1* nucleic acid be introduced into the plant cells, nor does it require that the introduced sequence be exactly identical to the *CUT1* nucleic acid. However, as with antisense suppression, the suppressive efficiency will be enhanced as (1) the introduced sequence is lengthened and (2) the sequence similarity between the introduced sequence and the endogenous *CUT1* gene is increased. Example I below provides an illustration of co-suppression of the endogenous *CUT1* gene by transformation of plants with the *CUT1* cDNA.

#### **d. Transformation and Regeneration Techniques**

Transformation and regeneration of both monocotyledonous and dicotyledonous plant cells is now routine, and the selection of the most appropriate transformation technique will be determined by the practitioner. The choice of method will vary with the type of plant to be transformed; those skilled in the art will recognize the suitability of particular methods for given plant types. Suitable methods may include, but are not limited to: electroporation of plant protoplasts; liposome-mediated transformation; polyethylene mediated transformation; transformation using viruses; micro-injection of plant cells; micro-projectile bombardment of plant cells; vacuum infiltration; and *Agrobacterium tumefaciens* (AT) mediated transformation. Typical procedures for transforming and regenerating plants are described in the patent documents listed at the beginning of this section.

#### **e. Selection of Transformed Plants**

Following transformation and regeneration of plants with the transformation vector, transformed

plants are preferably selected using a dominant selectable marker incorporated into the transformation vector. Typically, such a marker will confer antibiotic resistance on the seedlings of transformed plants, and selection of transformants can be accomplished by exposing the seedlings to appropriate concentrations of the antibiotic. Example I provides an example of such an approach in which seedlings  
5 were selected using kanamycin.

After transformed plants are selected and grown to maturity, they can be assayed to determine whether VLCFA synthesis has been altered as a result of the introduced transgene. This can be done in several ways, including, as described in Example 1, microscopic examination of the epicuticular wax layer and chromatographic analysis. Lipids may also be extracted from plant material and analyzed by  
10 gas chromatography as described by Dooner (1990). In addition, antisense or sense suppression of the endogenous *CUT1* gene may be detected by analyzing mRNA expression on Northern blots.

#### IX. Production of Sequence Variants

As noted above, modification of VLCFA synthesis in plant cells can be achieved by  
15 transforming plants with *CUT1* nucleic acids, antisense constructs based on *CUT1* nucleic acid sequences or other variants on *CUT1* nucleic acid sequences. With the provision of the *CUT1* cDNA and genomic sequences herein, the creation of variants on these *CUT1* nucleic acid sequences by standard mutagenesis techniques is now enabled.

Variant DNA molecules include those created by standard DNA mutagenesis techniques, for  
20 example, M13 primer mutagenesis. Details of these techniques are provided in Sambrook et al. (1989), Ch. 15. By the use of such techniques, variants may be created which differ in minor ways from the disclosed *CUT1* nucleic acids. DNA molecules and nucleotide sequences that are derivatives of those specifically disclosed herein and which differ from those disclosed by the deletion, addition or substitution of nucleotides while still encoding a protein which possesses the functional characteristic of  
25 the *CUT1* protein (i.e., very long chain fatty acid elongation activity) are comprehended by this invention. DNA molecules and nucleotide sequences which are derived from the *CUT1* nucleic acids include DNA sequences which hybridize under moderately stringent conditions to the DNA sequences disclosed, or fragments thereof.

Hybridization conditions resulting in particular degrees of stringency will vary depending upon  
30 the nature of the hybridization method of choice and the composition and length of the hybridizing DNA used. Generally, the temperature of hybridization and the ionic strength (especially the  $\text{Na}^+$  concentration) of the hybridization buffer will determine the stringency of hybridization. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook et al. (1989), chapters 9 and 11, herein incorporated by reference. By way of illustration  
35 only, a hybridization experiment may be performed by hybridization of a *CUT1*-derived probe (for example, the *CUT1* cDNA sequence) to a target DNA molecule (for example, the *CUT1* homolog from *Zea Mays*) which has been electrophoresed in an agarose gel and transferred to a nitrocellulose membrane by Southern blotting (Southern, 1975), a technique well known in the art and described in

(Sambrook et al., 1989). Hybridization with a target probe labeled with [<sup>32</sup>P]-dCTP is generally carried out in a solution of high ionic strength such as 6xSSC at a temperature that is 20-25°C below the melting temperature,  $T_m$ , described below. For such Southern hybridization experiments where the target DNA molecule on the Southern blot contains 10 ng of DNA or more, hybridization is typically carried out for 5 6-8 hours using 1-2 ng/ml radiolabeled probe (of specific activity equal to 10<sup>9</sup> CPM/μg or greater). Following hybridization, the nitrocellulose filter is washed to remove background hybridization. The washing conditions should be as stringent as possible to remove background hybridization but to retain a specific hybridization signal. The term  $T_m$  represents the temperature above which, under the prevailing ionic conditions, the radiolabeled probe molecule will not hybridize to its target DNA molecule. The  $T_m$  10 of such a hybrid molecule may be estimated from the following equation (Bolton and McCarthy, 1962):

$$T_m = 81.5^\circ\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%G+C) - 0.63(\% \text{ formamide}) - (600/l)$$

Where  $l$  = the length of the hybrid in base pairs.

15 This equation is valid for concentrations of  $\text{Na}^+$  in the range of 0.01 M to 0.4 M, and it is less accurate for calculations of  $T_m$  in solutions of higher  $[\text{Na}^+]$ . The equation is also primarily valid for DNAs whose G+C content is in the range of 30% to 75%, and it applies to hybrids greater than 100 nucleotides in length (the behavior of oligonucleotide probes is described in detail in Ch. 11 of Sambrook et al., 1989).

20 Thus, by way of example, for a 150 base pair DNA probe derived from the first 150 base pairs of the open reading frame of the *CUT1* cDNA (with a hypothetical %GC = 45%), a calculation of hybridization conditions required to give particular stringencies may be made as follows:

For this example, it is assumed that the filter will be washed in 0.3 xSSC solution following hybridization, thereby  $[\text{Na}^+] = 0.045\text{M}$ , %GC = 45%, Formamide concentration = 0,  $l = 150$  base 25 pairs,

$$T_m = 81.5 - 16(\log_{10}[\text{Na}^+]) + (0.41 \times 45) - (600/150)$$

and so  $T_m = 74.4^\circ\text{C}$ .

The  $T_m$  of double-stranded DNA decreases by 1-1.5°C with every 1% decrease in homology (Bonner et al., 1973). Therefore, for this given example, washing the filter in 0.3 xSSC at 59.4-64.4°C 30 will produce a stringency of hybridization equivalent to 90%. Alternatively, washing the hybridized filter in 0.3 xSSC at a temperature of 65.4-68.4°C will yield a hybridization stringency of 94%. The above example is given entirely by way of theoretical illustration. One skilled in the art will appreciate that other hybridization techniques may be utilized and that variations in experimental conditions will necessitate alternative calculations for stringency.

35 DNA sequences that encode a protein having VLCFA elongase activity and which hybridize to the disclosed *CUT1* nucleic acid sequences under hybridization conditions of at least 75%, more preferably at least 80%, 85% or 90% stringency, and most preferably at least 95% stringency are encompassed within the present invention.

The degeneracy of the genetic code further widens the scope of the present invention as it enables

major variations in the nucleotide sequence of a DNA molecule while maintaining the amino acid sequence of the encoded protein. For example, the fourth amino acid residue of the CUT1 protein is alanine. This is encoded in the *CUT1* ORF by the nucleotide codon triplet GCA. Because of the degeneracy of the genetic code, three other nucleotide codon triplets--GCT, GCC and GCG--also code for alanine. Thus, the nucleotide sequence of the *CUT1* ORF could be changed at this position to any of these three codons without affecting the amino acid composition of the encoded protein or the characteristics of the protein. Based upon the degeneracy of the genetic code, variant DNA molecules may be derived from the *CUT1* nucleic acid molecules disclosed herein using standard DNA mutagenesis techniques as described above, or by synthesis of DNA sequences. Thus, this invention also encompasses DNA sequences which encode the CUT1 protein but which vary from the *CUT1* nucleic acid sequences by virtue of the degeneracy of the genetic code.

One skilled in the art will recognize that DNA mutagenesis techniques may be used not only to produce variant DNA molecules, but will also facilitate the production of proteins which differ in certain structural aspects from the CUT1 protein, yet which proteins are clearly derivative of this protein and which maintain the essential characteristics of the CUT1 protein. Newly derived proteins may also be selected in order to obtain variations on the characteristic of the CUT1 protein, as will be more fully described below. Such derivatives include those with variations in amino acid sequence including minor deletions, additions and substitutions.

While the site for introducing an amino acid sequence variation is predetermined, the mutation *per se* need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed protein variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence as described above are well known.

Amino acid substitutions are typically of single residues; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e., a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. Obviously, the mutations that are made in the DNA encoding the protein must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure.

Substitutional variants are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 1 when it is desired to finely modulate the characteristics of the protein. Table 1 shows amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions.

Table 1.

	Original Residue	Conservative Substitutions
5	Ala	ser
	Arg	lys
	Asn	gln; his
	Asp	glu
	Cys	ser
10	Gln	asn
	Glu	asp
	Gly	pro
	His	asn; gln
	Ile	leu, val
15	Leu	ile; val
	Lys	arg; gln; glu
	Met	leu; ile
	Phe	met; leu; tyr
	Ser	thr
20	Thr	ser
	Trp	tyr
	Tyr	trp; phe
	Val	ile; leu

Substantial changes in enzymatic function or other features are made by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in protein properties will be those in which (a) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine.

The effects of these amino acid substitutions or deletions or additions may be assessed for derivatives of the CUT1 protein by analyzing the ability of the derivative proteins to catalyze the addition of C2 units to existing VLCFA units. These assays may conveniently be performed using the yeast-based systems for assaying fatty acid elongation described below.

#### X. Production of recombinant CUT1 protein using heterologous expression systems

Many different expression systems are available for expressing cloned nucleic acid molecules. Examples of prokaryotic and eukaryotic expression systems that are routinely used in laboratories are described in Chapters 16-17 of Sambrook et al. (1989), which are herein incorporated by reference. Such systems may be used to express CUT1 protein and derivatives at this protein at high levels to facilitate purification and functional analysis of the enzyme. Apart from permitting the activity of the enzyme to be



determined (which is particularly useful to assess the activity of homologous and derivative proteins), heterologous expression facilitates other uses of the purified enzyme. For example the purified enzyme produced by recombinant means may be used to synthesize VLCFAs and other fatty acid metabolites in vitro, particularly radio- or fluorescent- labeled forms of VLCFAs and metabolites. These molecules may be used as tracers to determine the location in plant tissues and cells of VLCFAs and their metabolites. The purified recombinant enzyme may also be used as an immunogen to raise enzyme-specific antibodies. Such antibodies are useful as both research reagents (such as in the study of VLCFA regulation in plants) as well as diagnostically to determine expression levels of the enzyme in agricultural products, including pollen.

By way of example only, high level expression of the CUT1 protein may be achieved by cloning and expressing the cDNA in yeast cells using the pYES2 yeast expression vector (Invitrogen, San Diego, CA). Secretion of the recombinant CUT1 from the yeast cells may be achieved by placing a yeast signal sequence adjacent to the CUT1 coding region. A number of yeast signal sequences have been characterized, including the signal sequence for yeast invertase. This sequence has been successfully used to direct the secretion of heterologous proteins from yeast cells, including such proteins as human interferon (Chang et al., 1986), human lactoferrin (Liang and Richardson, 1993) and prochymosin (Smith et al., 1985). Alternatively, the enzyme may be expressed at high level in standard prokaryotic expression systems, such as *E. coli*.

#### **XI. Assays for VLCFA elongase activity**

To aid the biochemical characterization of the CUT1 protein, or variants of this protein, the very long chain fatty acid elongase activity of the proteins may be determined by expressing the cDNA molecule which encodes protein in question in yeast. For that purpose, the full-length coding region of the cDNA may be linked to the galactose inducible GAL1 promoter in the *Saccharomyces cerevisiae* expression vector, pYES2 (Invitrogen). The yeast expressing the subject protein may then be employed to determine the substrate specificity of the CUT1 protein by one of the following approaches.

##### **a. In vitro assay for VLCFA elongase activity using cell-free yeast homogenate**

To determine the range of substrates recognized by the subject protein, acyl elongation activity is measured using substrates of varying carbon chain lengths and degrees of unsaturation. In each case, 15  $\mu$ M of an[1- $^{14}$ C]acyl CoA (C18, C20, C22, C24 in 0.005 % Triton X-100) is added to a standard assay mixture containing 80 mM Hepes-KOH, pH 7.2, 5 % glycerol, 1mM DTT, 0.5 mM NADPH, 1 mM ATP, 5 mM  $MgCl_2$ , 1 mM malonyl-CoA, and an aliquot of cell free extract (50  $\mu$ g protein) in a final volume of 50  $\mu$ L. Incubation is carried out at 30°C for 1 h. The reaction is stopped with 100  $\mu$ L of 4 N KOH in 80 % methanol and the lipids saponified for 1 h at 80°C. The mixture is then acidified by adding 100  $\mu$ L of cold 6N HCL and extracted twice with 500  $\mu$ L of cold hexane. The pooled hexane fractions are dried under  $N_2$ , followed by transmethylation for product analyses.

b. *In vivo* assay: Feeding of transformed yeast cells with radiolabelled acyl-tween substrates

A second approach for determining substrate specificity involves growth of yeast cells in the presence of various [ $1\text{-C}^{14}$ ]acyl-Tween substrates (C18, C20, C22, C24; Terzaghi, 1986). Fatty acyl substrates provided in the growth medium as Tween-fatty acid esters are readily taken up from the medium and used by the cells. For each FAE protein, yeast cells are initially grown in the presence of several concentrations of a single acyl-Tween substrate for different lengths of time to determine the optimal substrate concentration and the duration of the feeding assays. Once these parameters are established, yeast cells expressing the subject protein and control cells containing empty pYES2 plasmid are grown in a defined medium in the presence of a single radiolabelled acyl-Tween substrate. At the end of the experiment, cells are pelleted, and then resuspended in 1 mL of 1 N methanolic-HCl (Supelco). Treatment with methanolic-HCl converts fatty acids to methyl esters (FAME). Radiolabelled FAMES are analyzed as described below, to characterize the products generated by elongation of each acyl-Tween substrate. A comparison of radiolabelled FAMES from CUT1 containing yeast with FAMES isolated from control cells allows the determination of the elongation specificity of the subject FAE protein.

c. Product analyses

The products of the elongation assays obtained in (a), or pelleted yeast cells from experiment (b) are transmethyalted in a sealed tube using 1 N methanolic-HCl (Supelco) at 80°C for 1 h. Samples are then extracted twice with 500  $\mu\text{L}$  of hexane after the addition of 1 mL of 0.9% NaCl, and the pooled extracts containing FAMES concentrated under  $\text{N}_2$ . Radiolabelled FAMES are applied on  $\text{KC}_{18}$  reverse-phase TLC plates (Whatman), and separated in acetonitrile:tetrahydrofuran (85:15, v/v). Products of TLC separation are identified by co-chromatography with FAME standards, or by GC-MS. In addition, FAMES may be scraped from the TLC plates and their radioactivity determined by liquid scintillation counting.

EXAMPLES

The following examples serve to illustrate various applications of the present invention.

Example one: Modification of *A. thaliana* Wax Production By Transformation with the *CUT1* cDNA

a. Construction of binary transformation vector

The *CUTI* cDNA was cleaved out of the vector  $\lambda$ ZipLox (with *Kpn*I-*Bam*HI) and the resulting 1.85 kb fragment was directionally subcloned into the *Kpn*I-*Bam*HI sites of pGEM7z(f) (Promega, Madison, WI). The resulting plasmid was then fully cleaved with *Xho*I, but only partially cleaved with *Sst*I, (since the *CUTI* cDNA has an internal *Sst*I site). The 1.9 kb product was isolated on an agarose gel and directionally subcloned into the *Sal*I and *Sst*I sites of the vector pJD330 (Shaul and Galili 1992). This vector contains the 35S promoter of the cauliflower mosaic virus (CaMV) which provides constitutive expression in *Arabidopsis*. The subcloning results in the *CUTI* cDNA being inserted in a sense orientation with respect to the CaMV 35S promoter. The JD330-*CUTI* cDNA construct was ligated with pBIN19 and the resulting binary vector was designated p35S-*CUTI*. This binary vector was transformed into the *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986), and transformants were selected on LB medium containing 25  $\mu$ g/mL gentamycin and 50  $\mu$ g/mL kanamycin.

**b. Transformation of *Arabidopsis* with the p35S-*CUTI* transgene**

*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia was transformed using a combination of *in planta* (Chang et al., 1994, Katavic et al., 1994) and vacuum infiltration methods (Bechtold et al., 1993). Plants were grown until the primary inflorescence shoots reached 1-2 cm in height, and then these bolts were cut off. The wound site was inoculated with 50 mL of an overnight *Agrobacterium* culture harbouring the p35S-*CUTI* plasmid. After 4-6 days a number of secondary inflorescences that appeared were cut off, and vacuum infiltration was performed on these plants using the conditions described by Bechtold et al. (1993). Screening for transformed seed was done as described previously (Katavic et al., 1994). Briefly, seed from infiltrated plants were plated out (approximately 1500 seeds/plate) on solid minimal salts nutrient medium supplemented with 50  $\mu$ g/mL kanamycin. Seedlings that showed resistance were visible after approximately 8 days, because they turned green and elongated. Plants that were derived from seed harvested from different pots were considered as independent lines. Designations of transformed plants were as follows: the infiltrated plant--T1; primary transformants--T2; etc., as outlined in Katavic et al. (1994). Plants were grown at 20°C under continuous fluorescent illumination (100  $\mu$ Em<sup>2</sup>/s).

**c. *CUTI*-suppressed plants have altered wax composition**

Using the above transformation methods 46 kanamycin-resistant plants were obtained from seven different pots of *Arabidopsis*. Of the 46 plants obtained, 36 appeared waxless, having a glossy or *eceriferum* (*cer*) phenotype. At least one *cer* line was obtained from each pot implying that at least seven independent events had occurred in obtaining these lines. The surfaces of these *cer* plants were examined by a scanning electron (SE) microscope. SE micrographs clearly demonstrate that while wild-type plants were covered with the characteristic crystals of the epicuticular wax layer, transgenic *cer* plants were completely devoid of any wax crystals, implying that a severe *cer* phenotype has been created.

Plant tissue from the transgenic lines was analyzed for fatty acid composition. Plant tissue was immersed for 10 seconds in a 2:1 chloroform:methanol solution to remove surface waxes. Extracts were then evaporated to dryness under a stream of nitrogen. Waxes were dissolved in 100 µl of *N,O*-bis(Trimethylsilyl)trifluoroacetamide with 1% Trimethylchlorosilane (Pierce), and derivatized at 80 °C for 1 hour. Samples were analyzed in a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector, using either a DB-1 column or a DB-5 column.

GLC analyses were performed at the initial temperature of 150 °C, followed by a ramping of 4 °C/min to 320 °C, where it was held for 10 min. Peaks were identified by the comparison of retention times to reference standards, and mass spectrometry. Quantification was based on flame ionization detector peak areas, which were converted to mass units by comparison to the internal standard, 17:0-methylester, which was added to each sample prior to the extraction.

For wax load determinations only the principal surface lipids were measured, *n*-nonacosane (C29 alkane), 14- and 15-nonacosanol (C29 secondary alcohol), 15-nonacosanone (C29 ketone), C22-C30 aldehydes, C22-C30 primary alcohols and C16-C30 fatty acids (Hannoufa et al., 1993). The total area % of these peaks accounted for more than 90 % of the total area % of the sample.

The wax constituents that are found on the stems of *Arabidopsis* plants originate from two biosynthetic pathways (Figure 1). The decarbonylation pathway is the major pathway, which utilizes aldehydes to produce alkanes, secondary alcohols and ketones. In *Arabidopsis* (ecotype Columbia), the C29 species of the wax components produced by this pathway account for almost 90% of all the stem wax. The second pathway, the acyl-reduction pathway, produces primary alcohols, which account for approximately 5% of the total stem wax. Fatty acids and aldehydes, which are precursors for all the other wax components, are shared by both biosynthetic pathways and make up the remaining 5%.

Wax composition and quantity on the stems of wild-type and several transgenic lines were examined. Wild-type *Arabidopsis* stems contained on average 7106 (+/-) 1184 mg of wax/ g dry wt. In contrast, wax loads on the stems of all shiny *CUT1*-suppressed lines were severely reduced. For example, the wax load on the stems on the most severe line # 5 totals 483 (+/-) 83, only 6-7 % of the wild-type wax accumulation.

Analysis of wax composition of *CUT1*-suppressed plants revealed that the decarbonylation pathway is almost completely shut down. The C30 aldehyde, C29 alkane, C29 secondary alcohol and C29 ketone reach only 3.5 %, 2.2%, 1.4% and 2.2% of the levels found on wild-type plants, respectively. *CUT1*-suppression also has a major effect on the acyl-reduction pathway, causing a reduction in the levels of primary alcohols of over 50%. In addition, the relative abundance of different classes of alcohols is changed. C30 and C28 alcohols, the major alcohol species in wild type stems, have decreased by 90%, and C24 alcohol is the most abundant class in *CUT1* suppressed lines. The C24 species are also the most abundant classes of aldehydes and fatty acids in waxless transgenic plants. The described compositional changes were consistent in all 13 different *CUT1*-suppressed lines analyzed. These changes support the proposal that the role of the *CUT1* enzyme is elongation of the fatty acyl chain beyond 24 carbons.

**Example two: Production of conditionally male sterile *CUT1*-suppressed plants**

*CUT1*-suppressed *Arabidopsis* plants were produced as described in Example one and analyzed for male sterility. This analysis demonstrated that, in addition to stem and leaf wax synthesis, the *CUT1* gene product has an essential role in pollen development. Similar to *cer6-2* (Preuss et al., 1993) and *cer1* (Aarts et al., 1995) wax-deficient mutants of *Arabidopsis*, *CUT1*-suppressed plants are completely male sterile under normal growth conditions (30 to 40% relative humidity) although they produce normal amounts of pollen. However, when grown under high humidity (90 to 100%), pollen fertility is restored to the wild-type level, indicating that male sterility/fertility is conditional and environmentally controlled, just like in *cer6-2* and *cer1* mutants. For these two mutants, conditional male sterility is explained by alterations in the composition and content of the wax components of the tryphine layer covering the pollen grain. These long chain lipid molecules, produced in the tapetum layer of the anther, (Preuss et al., 1993) are needed in the tryphine for proper pollen-pistil signalling and pollen germination. Thus, in their absence, sterility occurs. Conditional male sterility is a valuable trait for plant breeders; being able to selectively inhibit self-fertilization of plants facilitates the production of hybrid plants. Accordingly, the *CUT1* cDNA and derivatives thereof may be useful in producing conditionally male sterile plants useful in breeding programs.

Taken together, the results of Examples one and two confirm that *CUT1* encodes a condensing enzyme that is involved in VLCFA biosynthesis of waxes which accumulate in the plant epidermis, as well as waxes required for the development of functional pollen grains. In addition the results show that transformation of plants using the *CUT1* cDNA is useful to produce plants having modified VLCFA compositions, as well as plants that exhibit conditional male sterility.

**Example three: Use of *CUT1* gene promoter sequence**

The promoter of the *CUT1* gene confers epidermis-specific expression. Accordingly, this promoter sequence may be used to produce transgene constructs that are specifically expressed in epidermal cells. Effective epidermis-specific expression may be achieved with less than the entire 1951 bases of sequence upstream of the *CUT1* ORF shown in Seq. I.D. No. 12. Thus, by way of example, epidermis-specific expression may be obtained by employing the 1209 base pair promoter fragment. One of skill in the art will recognize that still smaller regions of the sequence upstream of the *CUT1* ORF may be used to obtain epidermis-specific expression, such as a 50 base pair or 100 base pair region of the disclosed promoter sequence.

The determination of whether a particular sub-region of the disclosed sequence operates to confer effective epidermis-specific expression in a particular system (taking into account the plant species into which the construct is being introduced, the level of expression required, etc.) will be performed using known methods, such as operably linking the promoter sub-region to a marker gene (e.g. GUS), introducing such constructs into plants and then determining the level of expression of the marker gene in epidermis and other

plant tissues.

The present invention therefore facilitates the production, by standard molecular biology techniques, of nucleic acid molecules comprising this promoter sequence operably linked to a nucleic acid sequence, such as an open reading frame. Suitable open reading frames include open reading frames encoding any protein  
 5 for which epidermis-specific expression is desired.

Having illustrated and described the principles of isolating *CUT1* nucleic acids, the *CUT1* protein and modes of use of these biological molecules, it should be apparent to one skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all  
 10 modifications coming within the spirit and scope of the claims presented herein.

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## SEQUENCE LISTING

- (1) GENERAL INFORMATION
- (i) APPLICANT: The University of British Columbia
- 5 (ii) TITLE OF INVENTION: Nucleic Acids Encoding Plant Enzyme  
Involved In Very Long Chain Fatty Acid Synthesis
- (iii) NUMBER OF SEQUENCES: 12
- (iv) CORRESPONDENCE ADDRESS:
- 10 (A) ADDRESSEE: Sim & McBurney
- (B) STREET: 6th Floor, 330 University Avenue
- (C) CITY: Toronto
- (D) PROVINCE: Ontario
- (E) COUNTRY: Canada
- (F) POSTAL CODE: M5G 1R7
- 15 (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Disk, 3.5-inch
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: Windows 95
- (D) SOFTWARE: ASCII
- 20 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE: 14 April 1998
- (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- 25 (A) APPLICATION NUMBER: 60/043,831
- (B) FILING DATE: April 14, 1997
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE: April 10, 1998
- 30 (viii) PATENT AGENT INFORMATION
- (A) NAME: RAE, Patricia A.
- (B) REFERENCE/DOCKET NUMBER: 3055-18/PAR
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: (416) 595-1155
- 35 (B) TELEFAX: (416) 595-1163
- (2) INFORMATION FOR SEQ ID NO: 1:
- (i) SEQUENCE CHARACTERISTICS:
- 40 (A) LENGTH: 3712
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
- 45 TAGTGCTTTA TATATGTTTG ATACTTCTGT TTGGCAATAT CAATCATAGT 50
- AGAAAAGATA TGGACTTCAT TTGAGGTTTT TGGTGGATTG TGTCTATATG 100
- TGAAATCATG GGATCTCAAG ATTTGTCTGC ATTGAGTTTC CAAGTCAAAC 150
- 50 ATCGTAACTA CTGTTTGATT TTCCCTCATG CTTGCAGTTT TCATGGATAT 200
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	Phe	Glu	Ala	Met	Asp	Asp	Leu	Phe	Lys	Lys	Thr	Gly	Leu	Lys	
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15	CCT	AAA	GAC	GTC	GAC	ATC	CTT	ATC	GTC	AAC	TGC	TCT	CTT	TTC	2536
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20	TCT	CCC	ACA	CCA	TCG	CTC	TCA	GCT	ATG	GTC	ATC	AAC	AAA	TAT	2578
	Ser	Pro	Thr	Pro	Ser	Leu	Ser	Ala	Met	Val	Ile	Asn	Lys	Tyr	
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25	AAG	CTT	AGG	AGT	AAT	ATC	AAG	AGC	TTC	AAT	CTT	TCG	GGG	ATG	2620
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		225					230					235			
35	TTG	CTC	CAA	GTT	CAT	CCC	AAT	TCA	AAT	GCA	ATC	ATC	GTC	AGC	2704
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40	ACG	GAG	ATC	ATA	ACG	CCT	AAT	TAC	TAT	CAA	GGC	AAC	GAG	AGA	2746
	Thr	Glu	Ile	Ile	Thr	Pro	Asn	Tyr	Tyr	Gln	Gly	Asn	Glu	Arg	
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55	AAA	TAC	AAG	CTT	TCC	CAC	CTC	GTC	CGG	ACA	CAC	CGT	GGC	GCT	2872
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			310					315					320		
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	350					355						360						
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		365					370						375					
15	TAC	ATA	CCG	GAT	TTC	AAG	CTG	GCC	TTC	GAA	CAC	TTT	TGC	ATT	3124			
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			380					385						390				
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	420					425						430						
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		435					440					445						
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			450					455					460					
	AAC	TCT	GCC	GTG	TGG	AAA	TGT	AAC	CGT	ACG	ATT	AAG	ACA	CCT	3376			
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				465					470					475				
	AAG	GAC	GGA	CCA	TGG	TCC	GAT	TGT	ATC	GAC	CGT	TAC	CCT	GTC	3418			
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45					480					485								
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	Phe	Ile	Pro	Glu	Val	Val	Lys	Leu										
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(2) INFORMATION FOR SEQ ID NO: 2

10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1807

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Lys Leu Lys Tyr Val Lys Leu Gly Tyr Gln Tyr Leu Val Asn

15 20 25

30 CAT TTC TTG AGT TTT CTT TTG ATC CCG ATC ATG GCT ATT GTC 181

His Phe Leu Ser Phe Leu Leu Ile Pro Ile Met Ala Ile Val

30 35 40

GCC GTT GAG CTT CTT CGG ATG GGT CCT GAA GAG ATC CTT AAT 223

Ala Val Glu Leu Leu Arg Met Gly Pro Glu Glu Ile Leu Asn

35 45 50 55

GTT TGG AAT TCA CTC CAG TTT GAC CTA GTT CAG GTT CTA TGT 265

Val Trp Asn Ser Leu Gln Phe Asp Leu Val Gln Val Leu Cys

60 65

40 TCT TCC TTC TTT GTC ATC TTC ATC TCC ACT GTT TAC TTC ATG 307

Ser Ser Phe Phe Val Ile Phe Ile Ser Thr Val Tyr Phe Met

70 75 80

45 TCC AAG CCA CGC ACC ATC TAC CTC GTT GAC TAT TCT TGT TAC 349

Ser Lys Pro Arg Thr Ile Tyr Leu Val Asp Tyr Ser Cys Tyr

85 90 95

50 AAG CCA CCT GTC ACG TGT CGT GTC CCC TTC GCA ACT TTC ATG 391

Lys Pro Pro Val Thr Cys Arg Val Pro Phe Ala Thr Phe Met

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Glu His Ser Arg Leu Ile Leu Lys Asp Lys Pro Lys Ser Val



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	Glu Leu Ser Tyr Ile Glu Ser Lys Gly Arg Met Arg Arg Gly	
	435 440 445	
	GAT CGC GTT TGG CAA ATC GCG TTT GGG AGT GGT TTC AAG TGT	1441
	Asp Arg Val Trp Gln Ile Ala Phe Gly Ser Gly Phe Lys Cys	
	450 455 460	
45	AAC TCT GCC GTG TGG AAA TGT AAC CGT ACG ATT AAG ACA CCT	1483
	Asn Ser Ala Val Trp Lys Cys Asn Arg Thr Ile Lys Thr Pro	
	465 470 475	
50	AAG GAC GGA CCA TGG TCC GAT TGT ATC GAC CGT TAC CCT GTC	1525
	Lys Asp Gly Pro Trp Ser Asp Cys Ile Asp Arg Tyr Pro Val	
	480 485	
	TTT ATT CCC GAA GTT GTC AAA CTC TAA ACTGA	1557
	Phe Ile Pro Glu Val Val Lys Leu	

	AAACGTCTTT GAACGGTTTA GTAACGGTTT GATTTTGTGT TACGGTTTATG	1607
	GTTTATTTGG TCTCGGGATT TGGTTTAAAG GGGATTGAGA AATGGGAAGT	1657
5	TAGAGAGAAG AAAAAGCAAA GCATAAATGT TTGTATTTAA TTGCTCTGCA	1707
	TATACTTAAA TCTCTGCTTT TCATTGGGG TATTTTTTAG TTTCTCGTGC	1757
10	TGTAATTAAT AACTTGTGGT GTACTCAAAT AAGAATATTT CTCTCTGTTT	1807
	(2) INFORMATION FOR SEQ ID NO: 3:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1491	
15	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: double	
	(D) TOPOLOGY: linear	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:	
20	ATG CCT CAG GCA CCG ATG CCA GAG TTC TCT AGC TCG GTG	39
	Met Pro Gln Ala Pro Met Pro Glu Phe Ser Ser Ser Val	
	1 5 10	
	AAG CTC AAG TAC GTG AAA CTT GGT TAC CAA TAT TTG GTT AAC	81
25	Lys Leu Lys Tyr Val Lys Leu Gly Tyr Gln Tyr Leu Val Asn	
	15 20 25	
	CAT TTC TTG AGT TTT CTT TTG ATC CCG ATC ATG GCT ATT GTC	123
30	His Phe Leu Ser Phe Leu Leu Ile Pro Ile Met Ala Ile Val	
	30 35 40	
	GCC GTT GAG CTT CTT CGG ATG GGT CCT GAA GAG ATC CTT AAT	165
	Ala Val Glu Leu Leu Arg Met Gly Pro Glu Glu Ile Leu Asn	
	45 50 55	
35	GTT TGG AAT TCA CTC CAG TTT GAC CTA GTT CAG GTT CTA TGT	207
	Val Trp Asn Ser Leu Gln Phe Asp Leu Val Gln Val Leu Cys	
	60 65	
40	TCT TCC TTC TTT GTC ATC TTC ATC TCC ACT GTT TAC TTC ATG	249
	Ser Ser Phe Phe Val Ile Phe Ile Ser Thr Val Tyr Phe Met	
	70 75 80	
	TCC AAG CCA CGC ACC ATC TAC CTC GTT GAC TAT TCT TGT TAC	291
45	Ser Lys Pro Arg Thr Ile Tyr Leu Val Asp Tyr Ser Cys Tyr	
	85 90 95	
	AAG CCA CCT GTC ACG TGT CGT GTC CCC TTC GCA ACT TTC ATG	333
50	Lys Pro Pro Val Thr Cys Arg Val Pro Phe Ala Thr Phe Met	
	100 105 110	
	GAA CAC TCT CGT TTG ATC CTC AAG GAC AAG CCT AAG AGC GTC	375
	Glu His Ser Arg Leu Ile Leu Lys Asp Lys Pro Lys Ser Val	
	115 120 125	

	GAG TTC CAA ATG AGA ATC CTT GAA CGT TCT GGC CTC GGT GAG	417
	Glu Phe Gln Met Arg Ile Leu Glu Arg Ser Gly Leu Gly Glu	
	130 135	
5	GAG ACT TGT CTC CCT CCG GCT ATT CAT TAT ATT CCT CCC ACA	459
	Glu Thr Cys Leu Pro Pro Ala Ile His Tyr Ile Pro Pro Thr	
	140 145 150	
10	CCA ACC ATG GAC GCG GCT AGA AGC GAG GCT CAG ATG GTT ATC	501
	Pro Thr Met Asp Ala Ala Arg Ser Glu Ala Gln Met Val Ile	
	155 160 165	
15	TTC GAG GCC ATG GAC GAT CTT TTC AAG AAA ACC GGT CTT AAA	543
	Phe Glu Ala Met Asp Asp Leu Phe Lys Lys Thr Gly Leu Lys	
	170 175 180	
20	CCT AAA GAC GTC GAC ATC CTT ATC GTC AAC TGC TCT CTT TTC	585
	Pro Lys Asp Val Asp Ile Leu Ile Val Asn Cys Ser Leu Phe	
	185 190 195	
25	TCT CCC ACA CCA TCG CTC TCA GCT ATG GTC ATC AAC AAA TAT	627
	Ser Pro Thr Pro Ser Leu Ser Ala Met Val Ile Asn Lys Tyr	
	200 205	
30	AAG CTT AGG AGT AAT ATC AAG AGC TTC AAT CTT TCG GGG ATG	669
	Lys Leu Arg Ser Asn Ile Lys Ser Phe Asn Leu Ser Gly Met	
	210 215 220	
35	GGC TGC AGC GCG GGC CTG ATC TCA GTT GAT CTA GCC CGC GAC	711
	Gly Cys Ser Ala Gly Leu Ile Ser Val Asp Leu Ala Arg Asp	
	225 230 235	
40	TTG CTC CAA GTT CAT CCC AAT TCA AAT GCA ATC ATC GTC AGC	753
	Leu Leu Gln Val His Pro Asn Ser Asn Ala Ile Ile Val Ser	
	240 245 250	
45	ACG GAG ATC ATA ACG CCT AAT TAC TAT CAA GGC AAC GAG AGA	795
	Thr Glu Ile Ile Thr Pro Asn Tyr Tyr Gln Gly Asn Glu Arg	
	255 260 265	
50	GCC ATG TTG TTA CCC AAT TGT CTC TTC CGC ATG GGT GCG GCA	837
	Ala Met Leu Leu Pro Asn Cys Leu Phe Arg Met Gly Ala Ala	
	270 275	
55	GCC ATA CAC ATG TCA AAC CGC CGG TCT GAC CGG TGG CGA GCC	879
	Ala Ile His Met Ser Asn Arg Arg Ser Asp Arg Trp Arg Ala	
	280 285 290	
60	AAA TAC AAG CTT TCC CAC CTC GTC CGG ACA CAC CGT GGC GCT	921
	Lys Tyr Lys Leu Ser His Leu Val Arg Thr His Arg Gly Ala	
	295 300 305	
65	GAC GAC AAG TCT TTC TAC TGT GTC TAC GAA CAG GAA GAC AAA	963

	Asp	Asp	Lys	Ser	Phe	Tyr	Cys	Val	Tyr	Glu	Gln	Glu	Asp	Lys	
			310					315					320		
5	GAA	GGA	CAC	GTT	GGC	ATC	AAC	TTG	TCC	AAA	GAT	CTC	ATG	GCC	1005
	Glu	Gly	His	Val	Gly	Ile	Asn	Leu	Ser	Lys	Asp	Leu	Met	Ala	
			325					330					335		
10	ATC	GCC	GGT	GAA	GCC	CTC	AAG	GCA	AAC	ATC	ACC	ACA	ATA	GGT	1047
	Ile	Ala	Gly	Glu	Ala	Leu	Lys	Ala	Asn	Ile	Thr	Thr	Ile	Gly	
				340						345					
15	CCT	TTG	GTC	CTA	CCG	GCG	TCA	GAA	CAA	CTT	CTC	TTC	CTC	ACG	1089
	Pro	Leu	Val	Leu	Pro	Ala	Ser	Glu	Gln	Leu	Leu	Phe	Leu	Thr	
	350					355					360				
	TCC	CTA	ATC	GGA	CGT	AAA	ATC	TTC	AAC	CCG	AAA	TGG	AAA	CCA	1131
	Ser	Leu	Ile	Gly	Arg	Lys	Ile	Phe	Asn	Pro	Lys	Trp	Lys	Pro	
		365					370					375			
20	TAC	ATA	CCG	GAT	TTC	AAG	CTG	GCC	TTC	GAA	CAC	TTT	TGC	ATT	1173
	Tyr	Ile	Pro	Asp	Phe	Lys	Leu	Ala	Phe	Glu	His	Phe	Cys	Ile	
			380					385					390		
25	CAC	GCA	GGA	GGC	AGA	GCG	GTG	ATC	GAC	GAG	CTC	CAA	AAG	AAT	1215
	His	Ala	Gly	Gly	Arg	Ala	Val	Ile	Asp	Glu	Leu	Gln	Lys	Asn	
			395					400					405		
30	CTA	CAA	CTA	TCA	GGA	GAA	CAC	GTT	GAG	GCC	TCA	AGA	ATG	ACA	1257
	Leu	Gln	Leu	Ser	Gly	Glu	His	Val	Glu	Ala	Ser	Arg	Met	Thr	
				410						415					
35	CTA	CAT	CGT	TTT	GGT	AAC	ACG	TCA	TCT	TCA	TCG	TTA	TGG	TAC	1299
	Leu	His	Arg	Phe	Gly	Asn	Thr	Ser	Ser	Ser	Ser	Leu	Trp	Tyr	
	420					425					430				
	GAG	CTT	AGC	TAC	ATC	GAG	TCT	AAA	GGG	AGA	ATG	AGG	AGA	GGC	1341
	Glu	Leu	Ser	Tyr	Ile	Glu	Ser	Lys	Gly	Arg	Met	Arg	Arg	Gly	
		435					440				445				
40	GAT	CGC	GTT	TGG	CAA	ATC	GCG	TTT	GGG	AGT	GGT	TTC	AAG	TGT	1383
	Asp	Arg	Val	Trp	Gln	Ile	Ala	Phe	Gly	Ser	Gly	Phe	Lys	Cys	
			450					455					460		
45	AAC	TCT	GCC	GTG	TGG	AAA	TGT	AAC	CGT	ACG	ATT	AAG	ACA	CCT	1425
	Asn	Ser	Ala	Val	Trp	Lys	Cys	Asn	Arg	Thr	Ile	Lys	Thr	Pro	
			465					470					475		
50	AAG	GAC	GGA	CCA	TGG	TCC	GAT	TGT	ATC	GAC	CGT	TAC	CCT	GTC	1467
	Lys	Asp	Gly	Pro	Trp	Ser	Asp	Cys	Ile	Asp	Arg	Tyr	Pro	Val	
				480						485					
	TTT	ATT	CCC	GAA	GTT	GTC	AAA	CTC							1491
	Phe	Ile	Pro	Glu	Val	Val	Lys	Leu							

## (2) INFORMATION FOR SEQ ID NO: 4

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 497

(B) TYPE: amino acid

5 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```

10 Met Pro Gln Ala Pro Met Pro Glu Phe Ser Ser Ser Val Lys Leu Lys
    1           5           10           15
    Tyr Val Lys Leu Gly Tyr Gln Tyr Leu Val Asn His Phe Leu Ser Phe
    20           25           30
15 Leu Leu Ile Pro Ile Met Ala Ile Val Ala Val Glu Leu Leu Arg Met
    35           40           45
    Gly Pro Glu Glu Ile Leu Asn Val Trp Asn Ser Leu Gln Phe Asp Leu
    50           55           60
    Val Gln Val Leu Cys Ser Ser Phe Phe Val Ile Phe Ile Ser Thr Val

20 65           70           75           80
    Tyr Phe Met Ser Lys Pro Arg Thr Ile Tyr Leu Val Asp Tyr Ser Cys

    85           90           95
25 Tyr Lys Pro Pro Val Thr Cys Arg Val Pro Phe Ala Thr Phe Met Glu
    100          105          110
    His Ser Arg Leu Ile Leu Lys Asp Lys Pro Lys Ser Val Glu Phe Gln

    115          120          125
30 Met Arg Ile Leu Glu Arg Ser Gly Leu Gly Glu Glu Thr Cys Leu Pro

    130          135          140
    Pro Ala Ile His Tyr Ile Pro Pro Thr Pro Thr Met Asp Ala Ala Arg

    145          150          155          160
35 Ser Glu Ala Gln Met Val Ile Phe Glu Ala Met Asp Asp Leu Phe Lys

    165          170          175
    Lys Thr Gly Leu Lys Pro Lys Asp Val Asp Ile Leu Ile Val Asn Cys
    180          185          190
40 Ser Leu Phe Ser Pro Thr Pro Ser Leu Ser Ala Met Val Ile Asn Lys

    195          200          205
    Tyr Lys Leu Arg Ser Asn Ile Lys Ser Phe Asn Leu Ser Gly Met Gly

45 210          215          220
    Cys Ser Ala Gly Leu Ile Ser Val Asp Leu Ala Arg Asp Leu Leu Gln

    225          230          235          240
    Val His Pro Asn Ser Asn Ala Ile Ile Val Ser Thr Glu Ile Ile Thr

50 245          250          255
    Pro Asn Tyr Tyr Gln Gly Asn Glu Arg Ala Met Leu Leu Pro Asn Cys

    260          265          270

```

Leu Phe Arg Met Gly Ala Ala Ala Ile His Met Ser Asn Arg Arg Ser  
 275 280 285  
 5 Asp Arg Trp Arg Ala Lys Tyr Lys Leu Ser His Leu Val Arg Thr His  
 290 295 300  
 Arg Gly Ala Asp Asp Lys Ser Phe Tyr Cys Val Tyr Glu Gln Glu Asp  
 305 310 315 320  
 10 Lys Glu Gly His Val Gly Ile Asn Leu Ser Lys Asp Leu Met Ala Ile  
 325 330 335  
 Ala Gly Glu Ala Leu Lys Ala Asn Ile Thr Thr Ile Gly Pro Leu Val  
 340 345 350  
 15 Leu Pro Ala Ser Glu Gln Leu Leu Phe Leu Thr Ser Leu Ile Gly Arg  
 355 360 365  
 20 Lys Ile Phe Asn Pro Lys Trp Lys Pro Tyr Ile Pro Asp Phe Lys Leu  
 370 375 380  
 Ala Phe Glu His Phe Cys Ile His Ala Gly Gly Arg Ala Val Ile Asp  
 385 390 395 400  
 25 Glu Leu Gln Lys Asn Leu Gln Leu Ser Gly Glu His Val Glu Ala Ser  
 405 410 415  
 Arg Met Thr Leu His Arg Phe Gly Asn Thr Ser Ser Ser Ser Leu Trp  
 420 425 430  
 30 Tyr Glu Leu Ser Tyr Ile Glu Ser Lys Gly Arg Met Arg Arg Gly Asp  
 435 440 445  
 Arg Val Trp Gln Ile Ala Phe Gly Ser Gly Phe Lys Cys Asn Ser Ala  
 450 455 460  
 35 Val Trp Lys Cys Asn Arg Thr Ile Lys Thr Pro Lys Asp Gly Pro Trp  
 465 470 475 480  
 40 Ser Asp Cys Ile Asp Arg Tyr Pro Val Phe Ile Pro Glu Val Val Lys  
 485 490 495  
 Leu  
 497  
 45

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

50 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTGCTTTATA TATGTTTG 18

- (2) INFORMATION FOR SEQ ID NO: 6:
- (i) SEQUENCE CHARACTERISTICS:
- 5 (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
- 10 CGTCGGAGAG TTTTAATG 18
- (2) INFORMATION FOR SEQ ID NO: 7:
- (i) SEQUENCE CHARACTERISTICS:
- 15 (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
- 20 CTTCGATATC GGTGTGTTG 18
- (2) INFORMATION FOR SEQ ID NO: 8:
- (i) SEQUENCE CHARACTERISTICS:
- 25 (A) LENGTH: 24
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
- 30 AAATACCCTA ATCACATTTT GTAA 24
- (2) INFORMATION FOR SEQ ID NO: 9:
- (i) SEQUENCE CHARACTERISTICS:
- 35 (A) LENGTH: 24
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
- 40 TTTAAACAGA GAGAAATATT CTTA 24
- (2) INFORMATION FOR SEQ ID NO: 10:
- (i) SEQUENCE CHARACTERISTICS:
- 45 (A) LENGTH: 24
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
- 50 ATGCCTCAGG CACCGATGCC AGAG 24
- (2) INFORMATION FOR SEQ ID NO: 11:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24



(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

5 CAGCACGAGA AACTAAAAAA TACC 24

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 1951  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

15 TAGTGCTTTA TATATGTTTG ATACTTCTGT TTGGCAATAT CAATCATAGT 50  
 AGAAAAGATA TGGACTTCAT TTGAGGTTTT TGGTGGATTG TGTCTATATG 100  
 20 TGAAATCATG GGATCTCAAG ATTTGTCTGC ATTCAGTTTC CAAGTCAAAC 150  
 ATCGTAACTA CTGTTTGATT TTCCCTCATG CTTGCAGTTT TCATGGATAT 200  
 25 CTCAAGATTT GTCTTCTTGC ACTTTCCAAG TCAAACATAA AGTAACTACT 250  
 GATTGATATT CCCTCGTGTA TTACCCTCTT TCAAATGACA CAATTGGGCC 300  
 CAAGTAGAGG AATTTTCATAG TGAATTCAAA AGATTAACTG TATTCCACCG 350  
 30 TCGTATTTTG ATAACATTTA GTTATTCCTT TTCTTTTTTTT TCTTCTGCAA 400  
 CAGTTTTTTTT TTAATACATT TAGTGTTGGT TTGGTTCAAT GAAATATTAT 450  
 ATGTTACTTC TTTTTTTGGA AATAAATTAT TCATTCTTTC TACTATAAAA 500  
 35 GGAATTGTTC ATGCTTTTTT GATACAATAG TATACCATTT CAAAAGATAC 550  
 CATAGACCAG TTATTACATG AATCGCCAAA ACAACACTAA AATCAGAAAA 600  
 40 TCAGTATATT TTGGTATAGT CTCCAACATA CAATCATAAA ACCTCTGTGA 650  
 AATTTAAAT CTATATTTGA CATTTCAAAG TTTAACAACA TAGTTCTAAA 700  
 TAATTACCTA AATTTTAAGT CAAATGTGAA TTATATTTTA CTCTTCGATA 750  
 45 TCGGTTGTTG ACGATTAACC ATGCAAAAAA GAAACATTAA TTGCGAATGT 800  
 AAATAACAAA ACATGTAACCT CTTGTAGATA TACATGTATC GACATTTAAA 850  
 50 CCCGAATATA TATGTATACC TATAATTTCT CTGATTTTCA CGCTACCTGC 900  
 CACGTACATG GGTGATAGGT CCAAACCTCAC AAGTAAAAGT TTACGTACAG 950  
 TGAATTCGTC TTTTGGGTA TAAACGTACA TTTAATTTAC ACGTAAGAAA 1000

	GGATTACCAA TTCTTTCATT TATGGTACCA GACAGAGTTA AGGCAAACAA	1050
	GAGAAACATA TAGAGTTTGT ATATGTTTTT TTGGATAAAT ATTAAATTGA	1100
5	TGCAATATTT AGGGATGGAC ACAAGGTAAT ATATGCCTTT TAAGGTATAT	1150
	GTGCTATATG AATCGTTTCG CATGGGTACT AAAATTATTT GTCCTTACTT	1200
10	TATATAAACA AATTCCAACA AAATCAAGTT TTTGCTAAAA CTAGTTTATT	1250
	TGCGGGTTAT TTAATTACCT ATCATATTAC TTGTAATATC ATTCGTATGT	1300
	TAACGGGTAA ACCAAACCAA ACCGGATATT GAACTATTAA AAATCTTGTA	1350
15	AATTTGACAC AAATAATGA ATATCTAAAT TATGTTACTG CTATGATAAC	1400
	GACCATTTTT GTTTTGTAGA ACCATAATAT AAATTACAGG TACGTGACAA	1450
20	GTACTAAGTA TTTATATCCA CCTTTAGTCA CAGTACCAAT ATTGCGCCTA	1500
	CCGGGCAACG TGAACGTGAT CATCAAATCA AAGTAGTTAC CAAACGCTTT	1550
	GATCTCGATA AAATAAAAAG CTGACACGTC TTGCTGTTTC TTAATTTATT	1600
25	TCTCTTACAA CGACAATTTT GAGAAATATG AAATTTTAT ATCGAAAGGG	1650
	AACAGTCCTT ATCATTGCT CCCATCACTT GCTTTTGTCT AGTTACAAC	1700
30	GGAAATCGAA GAGAAGTATT AAAAAACAT TTTTCTCGTC ATTTATAAAA	1750
	AAATGACAAA AAATTAAATA GAGAGCAAAG CAAGAGCGTT GGGTGACGTT	1800
	GGTCTCTTCA TTAACCTCTC TCATCTACCC CTTCTCTGT TCGCCTTTAT	1850
35	ATCCTTCACC TTCCCTCTCT CATCTTCATT AACTCATCTT CAAAAATACC	1900
	CTAATCACAT TTTGTAACAA TAATACAATT ATACATTAAA ACTCTCCGAC	1950
40	G	1951

45

### Claims

We claim:

1. An isolated nucleic acid molecule that encodes a protein having very  
5 long chain fatty acid elongase activity, wherein the nucleic acid molecule is selected  
from the group consisting of:
  - (a) nucleic acids comprising at least 15 consecutive nucleotides of  
the sequence set forth in Seq. I.D. No. 3;
  - (b) nucleic acids possessing at least 70% sequence identity with the  
10 sequence set forth in Seq. I.D. No. 3; and
  - (c) nucleic acids that hybridize under conditions of at least 70%  
stringency with the sequence set forth in Seq. I.D. No. 3.
2. An isolated nucleic acid molecule according to claim 1 wherein the  
15 nucleic acid molecule comprises the sequence set forth in Seq. I.D. No. 3.
3. An isolated nucleic acid molecule according to claim 1 wherein the  
nucleic acid molecule possess at least 80% sequence identity with the sequence set  
forth in Seq. I.D. No. 3.  
20
4. An isolated nucleic acid molecule according to claim 1 wherein the  
nucleic acid molecule hybridizes under conditions of at least 80% stringency with the  
sequence set forth in Seq. I.D. No. 3.
- 25 5. A purified protein encoded by a nucleic acid molecule according to  
claim 1.
6. A recombinant vector comprising a nucleic acid molecule according  
to claim 1.  
30

7. A recombinant vector according to claim 6 wherein the nucleic acid molecule is in reverse orientation relative to an adjacent promoter sequence of the vector.
- 5 8. A transgenic plant comprising a recombinant vector according to claim 6.
9. A transgenic plant comprising a recombinant vector according to claim 7.
- 10 10. A transgenic plant comprising a recombinant expression cassette comprising a promoter sequence operably linked to a nucleic acid sequence selected from the group consisting of:
- 15 (a) nucleic acids comprising at least 15 consecutive nucleotides of the sequence set forth in Seq. I.D. No. 1;
- (b) nucleic acids possessing at least 70% sequence identity with the sequence set forth in Seq. I.D. No. 3; and
- (c) nucleic acids that hybridize under conditions of at least 70% stringency with the sequence set forth in Seq. I.D. No. 3.
- 20 11. A transgenic plant according to claim 10 wherein the nucleic acid sequence comprises at least 30 consecutive nucleotides of the sequence set forth in Seq. I.D. No. 1.
- 25 12. A transgenic plant according to claim 10 wherein the nucleic acid sequence possess at least 80% sequence identity with the sequence set forth in Seq. I.D. No. 3.
- 30 13. A transgenic plant according to claim 10 wherein the nucleic acid sequence hybridizes under conditions of at least 80% stringency with the sequence set forth in Seq. I.D. No. 3.

14. A transgenic plant according to claim 10, wherein the plant has a modified phenotype compared to a non-transgenic plant of the same species.

5 15. A transgenic plant according to claim 14 wherein the modified phenotype is a modified very long chain fatty acid composition.

16. A transgenic plant according to claim 15 wherein the modified phenotype is a modified epicuticular wax layer.

10 17. A transgenic plant according to claim 14 wherein the modified phenotype is modified seed oil composition.

15 18. A transgenic plant according to claim 14 wherein the modified phenotype is conditional male sterility.

19. A method of producing a plant with a modified very long chain fatty acid composition relative to a non-transgenic plant of the same species, comprising introducing into the plant a recombinant vector according to claim 6.

20 20. A transgenic plant produced by the method of claim 19.

21. A transgenic plant produced by sexual or asexual propagation of a plant according to claim 20 or the progeny of said plant.

25 22. An isolated nucleic acid molecule having a nucleotide sequence according to Seq. I.D. No. 3.

30 23. An isolated nucleotide that encodes a protein having an amino acid sequence as shown in Seq. I.D. No. 4.

24. A method of isolating a nucleic acid molecule encoding a plant very long chain fatty acid elongation enzyme, the method comprising hybridizing a nucleic acid preparation with a DNA molecule comprising at least 15 consecutive nucleotides of the sequence set forth in Seq. I.D. No. 1.

5

25. An isolated nucleic acid molecule isolated according to the method of claim 24.

26. A recombinant nucleic acid molecule comprising a promoter  
10 sequence operably linked to a nucleic acid sequence, wherein the promoter sequence comprises a CUT1 promoter.

27. A recombinant nucleic acid molecule according to claim 26 wherein  
the promoter sequence comprises at least 50 consecutive nucleotides of the sequence  
15 shown in Seq. I.D. No. 12.

28. A purified peptide having an amino acid sequence that is at least  
70% identical to the sequence set forth in Seq. I.D. No. 4.

1/1

## Arabidopsis Wax Biosynthesis

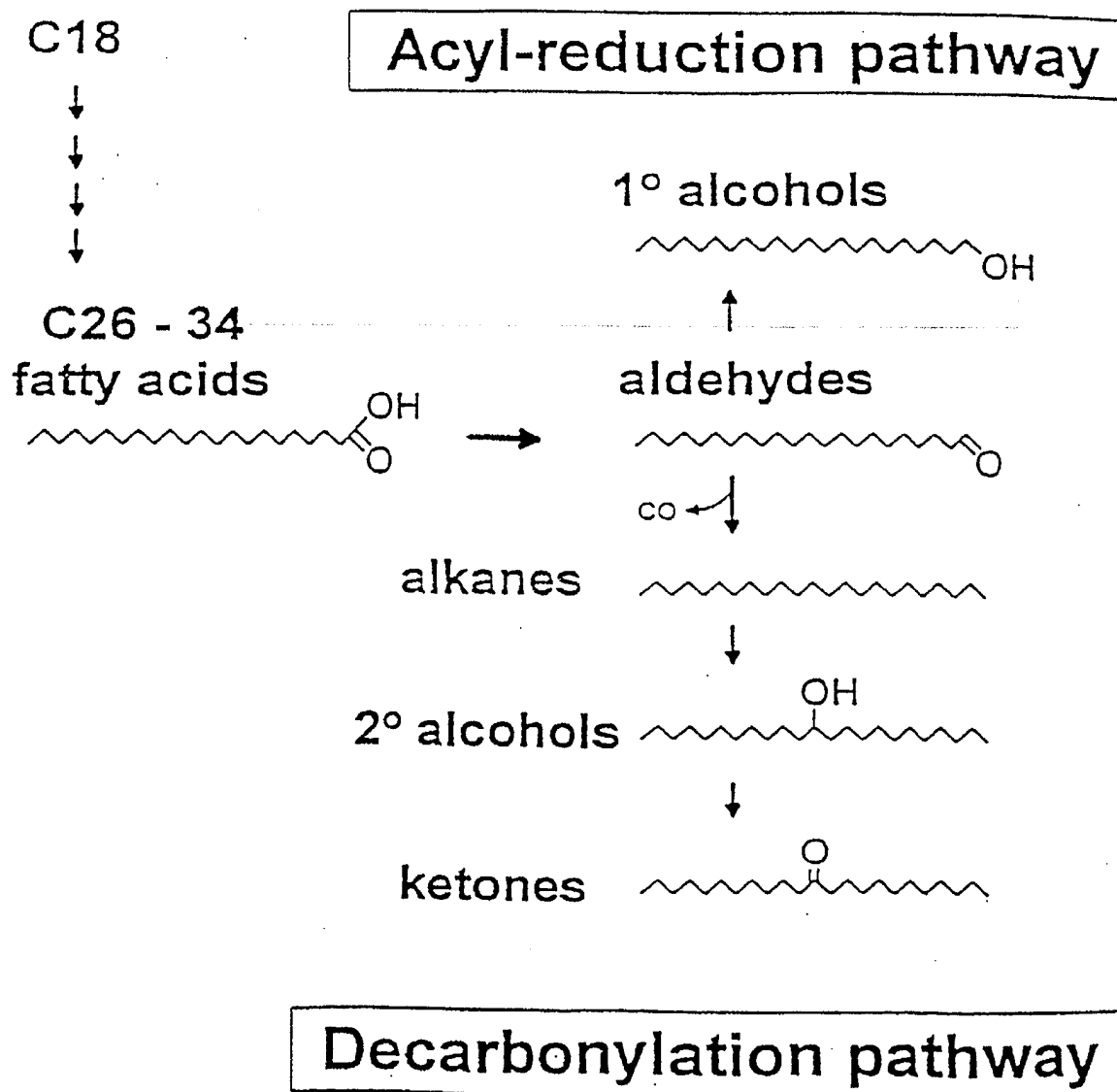


FIG. 1

# INTERNATIONAL SEARCH REPORT

In national Application No  
PCT/CA 98/00343

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C12N15/54 C12N15/11 C12N15/82 C12N9/10 A01H5/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N A01H		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEWMAN T. ET AL.: "AC T22193" EMBL DATABASE, 27 June 1994, HEIDELBERG, XP002072575 see the whole document ---	1, 3, 4, 6
X	NEWMAN T. ET AL.: "AC T76616" EMBL DATABASE, 25 March 1995, HEIDELBERG, XP002072576 see the whole document ---	1, 3, 4, 6
X	SOHAL A. AND JENKINS G.: "Epidermal-specific gene expression in Brassica and Arabidopsis" PLANT PHYSIOLOGY SUPPLEMENT, vol. 111, no. 2, June 1996, page 168 XP002072577 see abstract --- -/--	26
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : <div style="display: flex; justify-content: space-between;"> <div> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search  24 July 1998		Date of mailing of the international search report  05/08/1998
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer  Kania, T



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 98/00343

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 13582 A (DNA PLANT TECHN CORP) 9 May 1996 cited in the application see the whole document ----	1-28
A	WO 95 15387 A (CALGENE INC ;METZ JAMES GEORGE (US); LARDIZABAL KATHRYN DENNIS (US) 8 June 1995 cited in the application see the whole document ----	1-28
A	DE 44 33 307 A (NORDDEUTSCHE PFLANZENZUCHT HAN ;SAATVEREDELUNG LIPPSTADT BREME (DE) 21 March 1996 see the whole document ----	1-28
A	EVENSON K. AND POST-BEITTENMILLER D.: "Fatty acid-elongation activity in rapidly expanding leek epidermis" PLANT PHYSIOLOGY, vol. 109, 1995, pages 707-716, XP002072592 see the whole document -----	1-28

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Information on patent family members

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